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M.Sc Microbiology

IV- SEMESTER

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MICROBIAL BIOTECHNOLOGY

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UNIT-I MICROBIAL BIOTECHNOLOGY

Structure

1.1 INTRODUCTION

Microbial Biotechnology is an aspect of Biotechnology which involves the use of microorganisms for the production of microbial products that are clinically and economically important. Currently, it is referred as Industrial Microbiology. This technology involves the production of antibiotics, organic acids, organic solvents, amino acids, enzymes, vitamins, food products, beverages, single cell protein and pharmaceutical drugs.

1.2 OBJECTIVES

After reading this unit you will have a clear understanding about the field of Microbial Biotechnology, its scope and applications in human therapeutics, food production, agriculture, single cell protein and environment.

1.3 INTRODUCTION TO MICROBIAL BIOTECHNOLOGY

Microbial Biotechnology can be defined as a technology that uses microorganisms or derivatives to make products of industrial and medical applications. The microbial biotechnology can be simply referred as a fermentation process in which natural substrates of microbes are converted into value added products. The microorganisms exploited are natural, laboratory selected mutant or genetically engineered strains. In general, microorganisms are capable of producing an array of valuable compounds ranging from macromolecules such as proteins, nucleic acids and carbohydrates to smaller molecules called metabolites in small amounts. These metabolites produced by such microorganisms are further classified as primary and secondary metabolites. The primary metabolites are produced by the microbes during their energy metabolism and are essential for the vegetative growth, development and reproduction of the organisms. It includes carbohydrates, vitamins, proteins and amino acids. The secondary metabolites are produced after the growth of the microbes and usually during the stationary phase of the growth of the microorganism. It includes antibiotics, alkaloids and toxins. The microbial population produces such compounds for their own benefits, but those are exploited scientifically to produce products of potential application in human therapeutics and also for many other uses. This could be made possible through genetic engineering techniques. The insulin production is an example employing genetic engineering techniques by insertion of human insulin gene into bacterial cell. The microorganisms also have the ability to produce compounds that are not even produced in the laboratory. The microbial products such as enzymes, vitamins, alcohols, amino acids, recombinant proteins, fertilizers and biopesticides are of economically valuable with good market value. These products are cheaply produced by the application of microorganisms. For industrial production, the micro organisms are selected for their metabolic activities to produce one or more specific products in high rate. The microbes are also utilized in areas such as waste water treatment, Microbial Enhanced Oil Recovery (MEOR), biodegradation and biomining. With these aspects microbial biotechnology play an exemplary role in our lives and life spans.

1.3.1 Industrial Microorganisms

Microbes are used to synthesize a wide range of products that are highly valuable to human beings. Such microbes are industrially exploited to produce products such as food additives, beverages and therapeutics for human and animal health. The industrially exploited microorganisms are called as industrial microorganisms. It mostly

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includes bacteria and fungi. There are endless microbes that are used today at industrial level.

Table 1 Microorganisms employed for Production of Industrial Products

Products	**Microorganisms Employed**	**Microbial Product**
Antibiotics	*Streptomyces griseus* *Streptomyces parvullus*	*Streptomycin* Actinomycin D
Amino Acids	*Brevibacterium flavum* *Cornyebacterium glutamicum*	Lysine Glutamic Acid
Beverages	*Saccharomyces cerevisiae* *Saccharomyces uvarum*	Wine Beer
Organic Acids	*Lactobacillus* sp. *Gluconobacter suboxidans*	Lactic Acid Gluconic Acid
Vitamins	*lakeslea trispora* *Ashbya gossypii*	Lysine Glutamic Acid
Organic Solvents	*Saccharomyces cerevisiae* *Clostridium acetobutylicum*	Ethanol Acetone
Food Products	*Penicillium roquefortii* *Lactobacillus bulgaricus*	Cheese Yogurt
Enzymes	*Aspergillus oryzae* *Aspergillus niger*	Amylase Cellulase
Pharmaceutical Drug	*Trichoderma polysporum*	Cyclosporin A

1.3.2 Fermentation

Fermentation is defined as a metabolic process in which an organism converts sugar into alcohol. It is an anaerobic process which means it requires the absence of oxygen. Industrial fermentation refers to the chemical process involving microorganisms to obtain several products of human and animal need. It usually begins with suitable

microorganisms and maintenance of suitable conditions such as pH, oxygen control, foam control and temperature. Usually glucose is converted into pyruvic acid through glycolysis process. In the presence of oxygen, the pyruvic acid enters the TCA cycle and gets oxidized to produce 36-38 ATP molecules. But in the absence of oxygen, through alcoholic fermentation process, the pyruvic acid gets converted into ethanol and carbon dioxide. In lactic acid fermentation, it gets converted into lactate.

There are two main types of industrial fermentation techniques such as Solid State Fermentation (SSF) and Submerged Fermentation (SF).

Solid State Fermentation

This type of fermentation is carried out on a solid substrate such as saw dust, wheat bran and cereal grain. The solid substrates are the source of nutrients required for the growth of the microbes. This type of fermentation does not involve the usage of liquid medium (no water is required for the growth of the organism) but some moisture content is essential. Single pure cultures or mixed cultures may be used for this type of fermentation. Pretreatment of the raw material is carried out to increase the bioavailability of the nutrients for the microbes to act on them. Pretreatment techniques include soaking, boiling in water and other chemical treatments. It is mostly used for the production of fermented foods such as bread, yoghurt and cheese. The foods produced through this process are nutritious and easily digestible.

Examples of Solid State Fermentation

- Production of cheese by *Penicillium roqueforti*
- Production of α-amylase by *Aspergillus niger*
- Production of edible mushrooms such as *Agaricus* and *Pleurotus*. The solid substrate commonly used for this process is sawdust.

Submerged Fermentation

This type of fermentation technique involves the growth of the microorganisms as suspension on liquid medium called nutrient broth. It is also known as liquid fermentation and it utilizes substrates like molasses, soluble sugars, vegetable juices, sewage water and broths. It usually requires a high content of oxygen for the growth of the organisms and it is mostly suited for bacterial cells that require high moisture content. The microorganisms utilize the nutrients on the broth and produces bioactive compounds. The compounds are usually

secreted in the fermentation broth and are harvested by different techniques which is subjected to centrifugation and finally dried and packed. It is mostly exploited for industrial production of enzymes.

Examples of Submerged Fermentation

- Production of citric acid by *Aspergillus niger*
- Production of lactic acid by lactic acid bacteria

1.3.3 Fermentation Modes

Batch Fermentation

It is a closed culture system. The microorganisms are usually inoculated into a fixed volume of medium that supplies the essential nutrients for the growth of the microorganisms. As the growth phase of the microbe proceeds, there occurs depletion of the nutrients which is a disadvantage of the method. During the stationary phase, the microbes produce metabolites that are extracted from the fermenter through down streaming processes. The accumulation of metabolites also ceases the growth of the organisms in batch fermentation.

Fed Batch Fermentation

It is a modified method of batch fermentation. This fermentation is the most widely used one nowadays. In this fermentation, the substrate is added in increments several times during the fermentation process to increase the concentration of the biomass. Since the substrates are added at different periodic intervals, nutrient depletion is avoided during this process and hence the growth of the biomass is maintained at optimal level. The duration of the log and stationary phase of the growth of microorganisms is also high in this fermentation process and hence the production rate of the microbial products or secondary metabolites is also high. These are highly utilized in industrial processes.

Continuous Fermentation

In this type of fermentation, fresh nutrient medium is added into the fermenter continuously or in between during the course of the fermentation process. Likewise the used medium with the microorganisms are also subsequently removed from the fermenter tank for the recovery of the microbial product. High productivity is the major advantage of this technique. Volume of the medium and concentrations

of the nutrients are maintained at optimal level during the course of the fermentation process.

1.4 SCOPE & APPLICATION OF BIOTECHNOLOGY

1.4.1 In Human Therapeutics

Production of clinically important nutraceutical and pharmaceuticals by micro organisms and their derivatives is the major application of microbial biotechnology in human therapeutics. Production of recombinant protein and antibiotics for therapeutics is also an important application.

- Insulin, the first genetically engineered therapeutic agent is used to treat diabetes. It is expressed from human insulin genes on plasmid vector inserted into *E. coli*.
- Production of human growth hormone (hgH) for dwarf individuals is also an important application. The human growth hormone is secreted in the pituitary gland of humans. Reduction in the hormone level causes dwarfism. Genetically engineered hgH is cost effective and safe to use.
- Human tissue plasminogen activator is another therapeutic agent. It is proteolytic enzyme that cleaves single peptide bond in plasminogen to form plasmin. This plasmin has the capacity to degrade fibrin clots and hence it is widely used in the treatment of acute myocardial infarction.
- Production of DNA vaccines is another application of microbial biotechnology. It induces both humoral and cellular responses.
- The secondary metabolites produced by the microorganisms are utilized for the production of various antibacterial and anticancer drugs.
- Microorganism, *Streptomyces avermitilis* is used in the production of series of drug called Avermectin which is used to treat parasitic worms.
- Microorganisms are also industrially used for the production of vaccines, antibiotics and diagnostic kits. The *Streptococcus* sp. produces an enzyme called Streptokinase which acts as clot buster for removing blood clots.

1.4.2 In Agriculture

Microbial biotechnology plays an inevitable role in the field of agriculture. The microorganisms are widely exploited for the production of Biofertilizers and microbial pesticides, insecticides and herbicides.

- ✓ Biofertilizers are substances produced from microorganisms such as bacteria, fungi and algae. These microorganisms help the plants to absorb nutrients and fix atmospheric nitrogen into soil. Biofertilizers are cost effective, environmentally safe and increase crop production. Hence it is recommended over chemical fertilizers.
  - Bacterial Biofertilizers Eg: Rhizobium, Azospirillium and Azotobacter.
  - Fungal Biofertilizers Eg: *Mycorrhiza*
  - Algal Biofertilizers Eg: *Azolla*

- ✓ Microbial pesticides are composed of naturally occurring bacteria, virus and fungi and are used to control and kill pests that affect the plant growth. Generally prolonged use of chemical pesticides affects the soil fertility and imposes adverse effects on human beings and animals. In an alternative view, this microbial pesticide promotes soil fertility and is ecologically safe since it is produced from microorganisms.

- ✓ Microbial insecticides are also composed of naturally occurring microorganisms and are used to control insect pests. It is a part of Integrated Pest Management (IPM). Several species of *Bacillus* bacteria are commonly utilized for the production of insecticides. *Bacillus thuringiensis*, a Gram negative bacterium is widely used to kill insects and other arthropods. Virus like Baculovirus is utilized as a potential candidate for insecticide production. Entomopathogenic fungi is also employed for insecticide production which are host specific and in the process other beneficial insects do not get affected.

- ✓ Microbial herbicides are used to control the weeds that hinder the growth of the crop. The weeds affect the crop productivity and causes economical loss. In this approach, pathogens are isolated from weeds and are cultured to form infective propagules. These infective propagules when applied on the field targets and suppresses the growth of the target weeds.

1.4.3 In Food Technology

Microorganisms are used for the production of fermented foods and beverages. The fermenting property of the microorganisms makes it an ideal one for the production of food products such as cheese, bread and yoghurt.

Fermented Foods	Microorganism employed for Production
Bread	*Saccharomyces cerevisiae*
Cheese	*Penicillium roquefortii*
Yoghurt	*Lactobacillus bulgaricus*
Soy Sauce	*Aspergillus soyae*
Cakes	*Saccharomyces cerevisiae*

Table 1.1 Fermented Food Products and Microorganisms employed for their Production

The microorganisms used for the production influence the nature of the food like flavour and odour. The nutritive content of the fermented food is improved by the presence of microorganisms. Hence the fermented foods are highly nutritive and easily digestible.

1.4.4 In SCP

Single Cell Protein refers to the total protein extracted from microbial cell cultures. It serves as nutritional supplements for both humans and animals. It is rich in protein content but it also contains carbohydrates, vitamins and minerals. Hence it is considered as nutritionally rich supplement. Several microorganisms like bacteria, yeast, fungi and algae are utilized for the production of single cell protein. Substrates like molasses, whey, cellulose, animal manures are used for the production of SCP. The microorganism utilizes these substrates as nutrients required for their growth and produce increased biomass concentrations.

Microorganisms utilized for SCP production are as follows

- Bacteria*: Pseudomonas fluorescens*
- Algae: *Spirulina sp.*
- Fungi*: Aspergillus niger, Aspergillus fumigatus*
- Yeast*: Saccharomyces cerevisiae, Candida utilis*

Applications of SCP

1. It is used as supplemented food for undernourished children.
2. It prevents accumulation of cholesterol in the body.
3. It lowers blood sugar levels in diabetic patients.

1.4.5 In Environment

In Environmental concern, the microbial technological principles are applied in a variety of processes such as Bioremediation, Microbial Enhanced Oil recovery, Waste Water Management, Biosensors and Bioleaching

1.4.6 Bioremediation

It is the process of removal of pollutants from the environment through the application of microorganisms. The microorganisms utilized for the process are generally thermophilic anaerobic microorganisms and are used to remove organic wastes from the environment. The microorganisms degrade the environmental contaminants into less toxic forms through reactions that take place as a part of their metabolic process. Other interesting fact is that the microorganisms also possess some enzymes that allow the organism to utilize the contaminants as food for their growth. The effectiveness of the process depends on the environmental condition that allows microbial growth and activity.

1.4.7 Biomining

It is the process of cleaning up the sites that are polluted with metals. It can also be referred as the process of extracting economically valuable metals from rock ores and mine wastes. There are two methods employed in Biomining such as Bioleaching and Biooxidation. Bioleaching refers to the removal of metals from low grade ores and mineral concentrates. Usually the metals are found binded with the surface of the solid mineral and the microbes are able to oxidize that metal and make it dissolve in water. As the metals are dissolved in water, this method is called Bioleaching. This process is usually done with the application of microorganisms that are single celled and undergo chemosynthetic metabolism, for example mesophiles, the organisms that grows in moderate temperature and extremophiles, the organisms that grows in high pressure and temperature. Copper, zinc, cobalt and uranium are the metals extracted by this process. On the

other hand, some metals are not dissolved by the microbes. In such case, the minerals surrounding the metals are decomposed by the microbes allowing the recovery of metal directly from the site. This process also involves oxidation but difference here is that the metal of interest is solubilized.

1.4.8 Waste Water Treatment

Waste water treatment technology is also an application in which toxic materials such as textile dyes, heavy metals and pesticides are removed by the microorganisms. Diagnostics are developed to detect disease causing organisms in the water so as to ensure the water quality.

1.4.9 Biosensors

The genetically engineered microorganisms are exploited as living sensor to detect toxic chemicals in the soil, air and biological specimens. Toluene, a toxinogenic chemical found in chemical and radiation waste sites are also degraded by the application of microbial population.

1.5 CHECK YOUR PROGRESS QUESTIONS

1. Define Microbial Biotechnology.
2. What is Fermentation?
3. Abbreviate and define hgH.
4. Comment on Bioremediation.
5. What is Avermectin?

1.6 ANSWERS TO CHECK YOUR PROGRESS

1. Microbial Biotechnology is a branch of biotechnology which deals with the use of microorganisms for the production of clinically and economically important products to mankind and animal health.

2. Fermentation is a metabolic process usually an anaerobic process which involves the conversion of sugars into alcohols.

3. hgH refers to Human Growth Hormone. It is secreted by pituitary gland and is responsible for the growth of the organism.

4. Bioremediation is the process of removal of pollutants from the environment through the application of microorganisms.

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5. Avermectin is a drug used to treat parasitic worms. It is produced from *Streptomyces avermitilis*.

1.7 SUMMARY

- ✓ Microbial Biotechnology is a new emerging field of Biotechnology which involves the use of microorganisms for the production of therapeutic and industrial products of economic importance. With its application, it is now becoming an inevitable branch of Biotechnology.
- ✓ Several microorganisms are exploited for this process that may be natural, laboratory selected and genetically engineered ones.
- ✓ Usually the microbes produce these products as a part of their metabolic process and these are now exploited industrially for large scale production
- ✓ Fermentation is the basic for industrial biotechnology. It is the process of conversion of sugars into alcohols. Fermentations are usually carried out in fermenter.
- ✓ There are two types of fermentations such as Solid State Fermentation and Submerged Fermentation. As the name implies, solid medium is used in the former and liquid medium is used in the later one.
- ✓ Batch, Fed batch and Continuous fermentations are the modes of fermentation.
- ✓ In human therapeutics, it plays an important role in the production of insulin, human growth hormone and tissue plasminogen activator. These heterologous proteins are widely used in the treatment of diseases such as diabetes, dwarfism and acute myocardial infarction.
- ✓ In Agriculture, production of Biofertilizers, pesticides, insecticides and herbicides are its applications. These formulations are produced from microorganisms and are safe to humans and environment. These are cost effective too.
- ✓ In food industry, production of fermented foods such as bread, yoghurt and cheese form its applications.
- ✓ In environment, biomining and bioremediation are its applications.

1.8 KEYWORDS

SCP, Insulin, Diabetes, Pesticide, Nutrient Media.

1.9 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Write a brief note on Microbial Biotechnology.
2. Mention the industrially exploited microorganisms.
3. Differentiate Batch and Continuous fermentation
4. What do you know about SCP?
5. Mention the general scope of Microbial Biotechnology.

Long Answer Questions

1. Elaborate on the Fermentation Process.
2. Mention the applications of Microbial Biotechnology in human therapeutics.
3. Comment on the usage of microbes in agriculture.
4. Describe the scope of Microbial Biotechnology in environment.
5. Explain the production of SCP.

1.10 FURTHER READINGS

1. Alexander N. Glazer and Hiroshi Nikaido. 1995. Microbial Biotechnology: Fundamentals of applied Microbiology. Second Edition. Cambridge University Press.

2 Lee Yuan Kun. 2006. Microbial Biotechnology Principles and applications. Second Edition. World Scientific Publishing Co Pte Ltd.

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UNIT-II ALGAL BIOTECHNOLOGY

Structure

2.1 INTRODUCTION

2.1.1 Algae

Algae are very primitive aquatic plants of Kingdom Plantae. They don't have plant parts like roots, stems, leaves or fruits but have filamentous or non-filamentous thallus or body. Algal classification is based on the type of photosynthetic pigment present. Three classes of algae are Green, Brown and Red algae. Chlorophyll a and β-carotenes are present in all the three types but other photosynthesis pigments are different. Green algae contain chlorophyll b and xanthophylls; brown algae contain chlorophyll c and fucoxanthine; red algae have phycocyanin and phycoerythrin as pigments. Habitat of green algae (*Spirogyra*, *Oedogonium*) is fresh water or terrestrial whereas brown (*Sargassum*, *Laminaria*) and red algae (*Porphyra*, *Gelidium*) are marine

forms. The reserved food material of green algae is starch; mannitol and laminarin is present in brown algae; red algae have Floridian and starch as stored food. Blue Green Algae (BGA) or Cyanobacteria are excluded and placed in kingdom Monera as they possess prokaryotic cellular organization and functions Algae and Seaweeds possess many economically important species. Due to their structural heterogenity and other factors such as their irregular availability, naturally obtained algal polysaccharides may be unsuitable for certain pharmaceutical, food or similar applications, where a defined and consistent structure is required. Tissue cultures provide relatively uniform plant material at different organisational levels which can be maintained under defined chemical and physical conditions free from contaminating microorganisms and therefore are widely used in basic research. Consequently, tissue culture techniques could provide the opportunity for continuous production of a highly uniform product of interest in a factory setting.





Figure 1: Algae in Sea

The ability to obtain routinely callus culture, suspensions of cells or protoplasts from seaweeds and the subsequent development of an efficient tissue culture system for the production of uniform desired secondary metabolites would open new possibilities for pharmaceutical and food industries. An efficient tissue culture system and subsequent genetic improvement could also provide a basis for an intensive cultivation of new high yielding strains for the production of novel proteins, enzyme systems and genetic resources available to genetic engineers and the biotechnology industry. Tissue culture systems could also provide opportunities for research into growth and differentiation at all levels such as biochemistry, metabolism and molecular genetics studies of seaweeds.

2.2 OBJECTIVES

This unit provides the reader with information regarding biotechnological advances in algal management. This includes various

applications of cell fusion, tissue culture and hybridization process in algae. Finally the unit also covers important steps in generation of genetically modified algae with its application and risk management.

2.3 ALGAL BIOTECHNOLOGY

Algae are the most heterogeneous assemblage of autotrophic primitive plants and belong to the division Thallophyta. They are much diversified in form, function and structure and grow in almost all habitats. Most algal production is contributed to the detrital food chain either as particulate or dissolved organic matter and a small part is directly consumed by grazing. In the marine environment, algae form 90% of the total marine flora and thus playa very vital role in sustaining the productivity of the oceans. Among the algae in marine habitats, the microscopic, free-floating or swimming forms are known as phytoplanktons and the macroscopic, benthic forms are called seaweeds. Seaweeds generally grow abundantly on rocky substrata in intertidal and subtidal regions where adequate light for growth penetrates. Ever since the utilization of these resources for different purposes, several workers have investigated the possibilities of using these resources more effectively for the production of economically and commercially important products world-wide. Seaweeds have been primarily utilized as food in Japan and other Southeast Asian countries since ancient times. Porphyra, Laminaraia, Undaria and Monostroma are extensively cultivated for human consumption especially in Japan and China and are estimated to have a worldwide food value over US$ 1 billion annually (Abbott and Cheney, 1982). The second most important use of seaweeds is it being a source for extracting commercially important phycocolloids such as agar, carrageenan and alginates which have a world market value exceeding US$ 250 million annually. Besides these uses, seaweeds have also been considered as a source for the production of some pharmaceutically active compounds, fertilizers, bioflavors (Kajiwara *et al.*, 1989), and fodder and for energy (methane) production (Ryther and Hanisak, 1981). The increasing utilization of seaweeds coupled with the unavailability of sufficient raw material both for food and the production of phycocolloids eventually resulted in the successful development of different, efficient cultivation techniques as well as the domestication and selection of strains. In earlier studies, strain selection was done by simple screening of wild plants for desirable traits such as fast growth or rich chemical constituents. To date, most of the cultivated strains of Porphyra in Japan are selected by this simple selection method. However, recently as in agricultural crops, conventional spore breeding techniques have been applied to some economically important seaweeds to further improve their properties, both qualitatively and quantitatively. Presently, the classical example for genetic improvement of seaweeds is that of *Laminaria japonica* which was originally a cold-temperate seaweed and

which is now extensively cultivated in the subtropical waters of China (Fang *et al*., 1978; Tseng, 1981). Subsequently, successful intraspecific crosses were reported in Gigartina (Polanshek and West, 1977), Porphyra (Suto, 1963; Miura, 1979) and Eucheuma (Doty, 1979). Although these studies demonstrated the feasibility of sexual hybridization in certain genera, the principal difficulty of this approach appears to be the existence of sexual incompatibility and interfertility between different species (Chen and Taylor, 1980; Guiry and West, 1983). Consequently, somatic hybridization and cell transformation techniques using protoplasts have been developed as an alternative and to overcome some of the difficulties encountered in conventional breeding studies. It has also been suggested (Cheney 1984, 1990) that to make seaweed cultivation commercially more attractive, it is necessary to establish improved strains having valuable, new or improved products which are not available in the existing wild plants or commercially existing valuable products must be produced at reduced costs, increased quality on greater dependability. The work on various aspects of the isolation, culture, and fusion of algal protoplasts and the production of somatic hybrids is reviewed in this chapter.

(1) Algal Growth in Laboratory

In laboratory, algae are cultivated under aseptic conditions and controlled physical parameters like pH, temperature, light intensity, shaking of growth medium and proper incubation time. Algae being photoautotrophic are grown in broth or agar medium supplemented with micronutrients like magnesium, nitrate, calcium and iron. The incubation is always carried out in illuminated growth chamber for about 7-15 days till algal growth is visualized. Identification is carried out with the help of standard classification manual.

(2) Algal Farming

Algal cultivation is cheap as compared to other economic crops. Algae do not require prepared and fertilized land for their growth. They can grow on marginal land like salinated or drought affected hard soils. They also grow in waste water effluents or sewage or even waste water from nuclear reactors. They can be grown in open ponds or bioreactor tanks or closed ponds or tanks covered inside shade nets. Algae are cultivated on large scale in photo bioreactors. Reactors are plastic pumps containing nutrient water for algal growth. Carbon dioxide is supplied intermittently to enhance algal biomass. Algae when grown in closed system are protected from air borne microbial contamination particularly fungal spores which can be pathogenic to algae.

(3) Collection, Storage and Preservation

Algae grow in almost every habitat in every part of the world. They can be found on very different natural substrates from animals (snails, crabs, sloths and turtles are algal hosts) to plants (tree trunks, branches and leaves, water plants and macroalgae) from springs and rivers to hypersaline lagoons and salt lakes. They also colonize artificial habitats such as dams and reservoirs, fountains and pools, but cans, bottles, plant pots or dishes allow algae to extend their natural range. The ubiquities of these organisms together with the plasticity of their metabolic requirements make many algal species easily available for investigation, collection or simple observation.

Floating microalgae can be collected with a mesh net with 25-30 µm pores or if in sufficient quantity (i.e., colouring the water) by simply scooping a jar through the water. A small amount of the bottom sediments will also provide many of the algal species that live in or on these sediments. Some algae live attached to other types of substrates such as dead leaves, twigs and any underwater plants which may be growing in the water. Macroalgae and the attached microalgae can be collected by hand or with a knife including part or all of the substrate (rock, plant, wood, etc.) if possible. Algae growing on soil are difficult to collect and study, many requiring culturing before sufficient and suitable material are available for identification.

Any sample should be labelled with standard information such as the locality, date of collection and as many of the following features as possible: whether the water is saline, brackish or fresh; whether the collection site is terrestrial, a river, a stream or a lake; whether the alga is submerged during water level fluctuations or floods; whether the water is muddy or polluted; whether the alga is free floating or attached and if the latter, the type of substrate to which it is attached and the color, texture and size of the alga. Algae can be stored initially in a glass jar, plastic bottle or bag or in a vial with some water from the collecting site. The container should be left open or only half filled with liquid and wide shallow containers are better than narrow deep jars. If refrigerated or kept on ice soon after collecting, most algae can be kept alive for short periods for about a day or two. If relatively sparse in the sample, some algae can continue to grow in an open dish stored in a cool place with reduced light. For long-term storage, specimens can be preserved in liquid, dried or made into a permanent microscope mount. Even with ideal preservation, examination of fresh material is sometimes essential for an accurate determination. Motile algae particularly must be examined

while flagella and other delicate structures remain intact as any kind of preservation procedure causes the detachment of the flagella.

Commercial formalin (40% formaldehyde in water), diluted between 1/10 and 1/20 with the collecting solution is the most commonly used fixative. As formaldehyde is considered carcinogenic, any contact with skin, eyes and air passages should be avoided. This compound mixed with other chemicals such as glacial acetic acid and alcohol (FAA 1:1:8 by volume: 40% formaldehyde 1:glacial acetic acid 1:95% alcohol 8) gives better preservation results for some of the more fragile algae whereas the standard alcohol and water mix (e.g., 70% ethyl alcohol) will ruin all but the larger algae. However, FAA may cause thin-walled cells to burst. Colour is an important taxonomic characteristic especially for Cyanobacteria and formalin is a good preservative for green algae, cyanobacteria and dinoflagellates because cell colour remains intact if samples are stored in the dark. Algae can be kept in diluted formalin for a number of years but the solution is usually replaced by 70% ethyl alcohol with 5% glycerine, the latter to prevent accidental drying out.

Lugol's solution is the preferred preservative commonly used for short-term storage of microalgae for about few months, possibly a year or more. It is excellent for preserving chrysophytes but it makes the identification of dinoflagellates difficult, if not impossible. Samples can be preserved and kept in dark bottles away from light for as long as 1 year in Lugol's solution (0.05-1% by volume). The solution is prepared by dissolving 20 g of potassium iodide and 10 g of iodine crystals in 180 ml of distilled water and by adding 20 ml glacial acetic acid. The Lugol's solution has a shelf life that is affected by light of about 6 months.

Dried herbarium specimens can be prepared by "floating out" similar to aquatic flowering plants. Ideally, fresh specimens should be fixed prior to drying. Most algae will adhere to absorbent herbarium paper. Smaller, more fragile specimens or tangled, mat-forming algae may be dried onto mica or cellophane. After "floating out," most freshwater algae should not be pressed but simply left to air dry in a warm dry room. If pressed, they should be covered with pieces of waxed paper, plastic or muslin cloth so that the specimen does not stick to the drying paper in the press.

To examine a dried herbarium specimen, a few drops of water has to be added to the specimen to make it swell and lift slightly from the paper. This makes it possible to remove a small portion of the specimen with forceps or a razor blade.

Observations preferably including drawings or photographs based on living material are essential for the identification of some genera and a valuable adjunct to more leisurely observations on preserved material for others. The simplest method is to place a drop of the water including the alga onto a microscope slide and carefully lower a coverslip onto it. It is always tempting to put a large amount of the alga onto the slide but smaller fragments are much easier to view under a microscope. Microalgae may be better observed using the "hanging drop method" with a few drops of the sample liquid being placed on a coverslip which turns over onto a ring of paraffin wax, liquid paraffin or a "slide ring."

A permanent slide can be prepared with staining such as aniline blue (1% aqueous solution, pH 2.0-5), toluidine blue (0.05% aqueous solution, pH 2.0-2.5) and potassium permanganate, $KMnO_4$ (2% aqueous solution) which are useful stains for macroalgae. Different stains suit for different species and India Ink which is a good stain for highlighting mucilage and some flagella-like structures. Staining time ranges from 30sec to 5 minutes depending on the material after which the sample is to be rinsed in water. Mounting is achieved by adding a drop or two of glycerine solution (75% glycerine, 25% water) to a small piece of the sample placed on a microscope slide by carefully lowering the coverslip. Sealing with nail polish is essential. This method is unsuitable for most unicellular algae which should be examined fresh or in temporary mounts of liquid-preserved material.

Magnifications between 40 and 1000 times are required for the identification of all but a few algal genera. A compound microscope with 10X- 12X eyepiece and 4X-10X-40X objectives is therefore an essential piece of equipment for anyone wishing to discover the world of algal diversity. An oil immersion 100X objective would be a useful addition particularly when aiming at identifying to species level. Phase-contrast or interference (e.g., Nomarski) microscopy can improve the contrast for bleached or small specimens. A dissecting microscope providing 20X, 40Xupto 60X magnifications is a useful aid but is secondary to a compound microscope. A camera lucida attachment is helpful for producing accurate drawings while an eyepiece micrometer is important for size determinations. Formulas for calculating biomass for various phytoplankton shapes using geometric forms and measurements and shape code for each taxa exists in the literature and are routinely used in the procedure for phytoplankton analysis that require biovolume calculations. Moreover, software packages for image recognition and analysis are available which can process phytoplankton images acquired by means of a TV camera mounted onto a compound microscope. Scanning and Transmission electron microscopes are

beyond the reach of all but with special institutions, however, they are an essential tool for identifying some of the very small algae and investigating the details of their ultrastructure.

2.3.1 Culture Types

A culture can be defined as an artificial environment in which the algae grow. In theory, culture conditions should resemble the alga's natural environment as far as possible; in reality many significant differences exist, most of which are deliberately imposed. In fact, following isolation from the natural environment, algal strains are maintained under largely artificial conditions of media composition, light and temperature. The imposition of an artificial environment on a cell population previously surviving under complex, fluctuating conditions and following a seasonal life cycle inevitably causes a period of physiological adaptation or selection during which population growth will not occur or is very slow.

While contaminated algal cultures which have previously been satisfactory for certain application and experiments, modern experimental methods and applications demand that contaminants are not generally present and that the taxonomy and growth characteristics of strains are defined. Hence, for most purposes, algal cultures are maintained as unialgal, contaminant-free or axenic stocks. "Unialgal" cultures contain only one kind of alga usually a clonal population (but which may contain bacteria, fungi, or protozoa), whereas "axenic" cultures should contain only one alga and no bacteria, fungi or protozoa.

To obtain a unialgal culture, one species must be isolated from all the rest; three major techniques borrowed from microbiology are available for obtaining unialgal isolates: streaking and successive plating on agar media, serial dilution and single-cell isolations using capillary pipettes. Streaking is useful for single-celled, colonial or filamentous algae that will grow on an agar surface. Filaments can be grabbed with a slightly curved pipette tip and dragged through soft agar (less than 1%) to remove contaminants. It is best to begin with young branches or filament tips that have not yet been extensively epiphytized.

Many flagellates, however as well as other types of algae must be isolated by single-organism isolations or serial-dilution techniques. A particularly effective means of obtaining unialgal cultures is isolation of zoospores immediately after they have been released from parental cell walls, but before they stop swimming and attached to a surface. Recently released zoospores are devoid of contaminants,

unlike the surfaces of most algal cells, but catching zoospores requires a steady hand and experience.

Sterile cultures of microalgae may be obtained from specialized culture collections. Alternatively, axenic cultures can be obtained by treating isolated algae to an extensive washing procedure or with one or more antibiotics. Resistant stages such as zygotes or akinetes can be treated with bleach to kill epiphytes and later planted on agar for germination. It is usually necessary to try several different concentrations of bleach and times of exposure to find a treatment that will kill epiphytes without harming the alga. When diatoms represent the contaminating species, addition of low concentrations (5mg/l) of germanium dioxide, GeO_2 to a culture medium can inhibit diatom growth as it disrupts silica deposition.

“Cleaning” previously contaminated cultures is a skillful and time-consuming process and could take several years in sizeable collections. Extensive measures must be taken to keep pure unialgal cultures chemically and biologically clean. Chemical contamination may have unquantifiable often deleterious and therefore undesirable effects on algal growth. Biological contamination of pure algal cultures by other eukaryotes and prokaryotic organisms in most cases invalidates experimental work and may lead to the extinction of the desired algal species in culture through outcompetition or grazing. In practice, it is very difficult to obtain bacteria-free (axenic) cultures and although measures should be taken to minimize bacterial numbers, a degree of bacterial contamination is often acceptable.

If biological contaminants appear in a culture, the best remedy is to isolate a single cell from the culture with a micropipette and try to establish a new, clean clonal culture. Alternatively, the culture can be streaked on an agar plate in the hope of attaining a colony free of contaminants. Neither of these methods works well, however, for eliminating bacteria that attach firmly to the surface of microalgae. Placing a test-tube of microalgal culture in a low-intensity 90 kilocycles sec^{-1} ultrasonic water bath for varying lengths of time for few seconds to tens of minutes can sometimes physically separate bacteria without killing the algae making it easier to obtain an axenic culture by micropipette isolation. Often, however, to achieve an axenic culture, antibiotics must be added to the growth medium to discourage growth of contaminating cyanobacteria and other bacteria. Best results appear to occur when an actively growing culture of algae is exposed to a mixture of penicillin, streptomycin and gentamycin for around 24 h. This drastically reduces the growth of bacteria while allowing the microalgae to continue to grow, increasing the chances of obtaining an axenic cell when using micropipette or agar streaking

isolation. Different algal species tolerate different concentrations of antibiotics and hence a range of concentrations should be used generally from 50-500% w/v). Other antibiotics that can be used include chloramphenicol, tetracycline and bacitracin. Antibiotic solutions should be made with distilled water and filter-sterilized (0.2 µm filter units) into sterile tubes and should be stored frozen until use. Another approach is to add a range of antibiotic concentrations to a number of subcultures and then to select the culture that has only surviving algal cells with no surviving bacteria or other contaminants. Sterility of cultures should be checked by microscopic examination (phase contrast) and by addition of a small amount of sterile bacterial culture medium (e.g., 0.1% peptone) to a microalgal culture and observing regularly for bacterial growth. Absence of bacterial growth does not however ensure that the microalgal culture is axenic, because the majority of bacteria do not respond to standard enrichments. In reality there is no way of demonstrating that a microalgal culture is completely axenic. In practice, therefore, axenic usually means "without demonstrable unwanted prokaryotes or eukaryotes." Some microalgal cultures may die when made axenic probably due to the termination of obligate symbiotic relationships with bacteria.

The collection of algal strains should be carefully protected against contamination during handling and poor temperature regulation. To reduce risks, two series of stocks are often retained, one which supplies the starter cultures for the production system and the other which is only subjected to the handling necessary for maintenance. Stock cultures are kept in test-tubes at a light intensity of about 1.5 Wm^2 and a temperature of 16-19°C. Constant illumination is suitable for the maintenance of flagellates, but may result in decreased cell size in diatom stock cultures. Stock cultures are maintained for about a month and then transferred to create a new culture line.

2.3.1.1 Decontamination of Seaweed Tissue

The isolation of axenic rather than merely unialgal, material and the subsequent maintenance of sterility is common to all tissue culture procedures. Tissues completely free from biological contaminants are essential because nutrient media often support the growth of contaminants. As a result, the contaminants may overgrow the culture or produce metabolites which may influence the growth and metabolic responses of the cultured material and modify applied treatments.

Tissue culture is the growth of tissues or cells in an artificial medium facilitating the separation from the organism. This is typically facilitated via use of a liquid, semi-solid or solid growth medium such

as broth or agar. Tissue culture commonly refers to the culture of animal cells and tissues with the more specific term plant tissue culture being used for plants and algae cells and tissues.

2.3.1.2 Secondary Decontamination of Cell Lines

The culture sterility effect after decontamination of the original explants may only be temporary. Tait *et al*., (1990) established callus cultures from sterile discs of *Porphyra umbilicalis*. After about a month of the culture, all the cell lines proved to have bacterial contamination. To eliminate the contamination, the aggregates were inoculated into liquid growth medium supplemented with antibiotic mixture: Kanamycin (100 mg/l) and Neomycin (50 mg/l) for 42-72 days. Successful secondary decontamination was also achieved through 30 days culture of the callus on semi-solid medium supplemented with Penicillin G (39 mg/l), Coltrimazole (1.7 mg/l) and Germanium dioxide (6 mg/l).

Antibiotics are useful surface sterilants when secondary contamination occurs as they provide a broad spectrum of antibacterial activity with minimal toxicity to the plant cells (Pollock *et al*., 1983).

2.3.2. Cell Fusion

Cell fusion is an important cellular process in which several uninuclear cells (cells with a single nucleus) combine to form a multinuclear cell known as a syncytium. Cell fusion occurs during differentiation of muscle, bone and trophoblast cells during embryogenesis and during morphogenesis. Cell fusion is a necessary event in the maturation of cells so that they maintain their specific functions throughout growth.

2.3.2.1. Fusion Methods

The fusion can be described by three consecutive phases: 1) placing the protoplasts in close contact 2) disruption and fusion in a limited and localized place in the adjacent membranes and 3) formation of bridges between protoplasts which allows cytoplasmic continuity between the cells (Navratilova, 2004). The enzymatic degradation of the walls reduces constrictions in the plasmodesma in vegetative an algal tissues as a result the plasmogamy between the neighbouring protoplasts can occur spontaneously (Withers & Cocking, 1972). Normally, isolated protoplasts do not tend to fuse with each other because their surface has a negative charge ranging between -10 mV and -30 mV outside the plasma membrane which results in a strong tendency to repel each other. Based on that, fusion requires a chemical

inducer agent or a system that reduces the electronegativity of the isolated protoplasts and allows them to have a closer contact (Navratilova, 2004; Verma et *al.*, 2000). Chemofusion and electro-fusion are the major alternatives for protoplast fusion. Additionally, nuclear fusion through microinjection technique can also be explored. This last method consists in introducing the foreign material into the cell by the insertion of a glass capillary in the intracellular environment (Zhou *et al*., 2017).

2.3.3. Tissue Culture

(a) Culture Parameters

A culture has three distinct components: a culture medium contained in a suitable vessel; the algal cells growing in the medium and air to allow exchange of carbon dioxide between medium and atmosphere.

For an entirely autotrophic alga, all that is needed for growth is light, CO_2, water, nutrients and trace elements. By means of photosynthesis, the alga will be able to synthesize all the biochemical compounds necessary for growth. Only a minority of algae is however entirely autotrophic; many are unable to synthesize certain biochemical compounds like certain vitamins and require these to be present in the medium especially in an obligate mixotropy condition.

The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature. The most optimal parameters as well as the tolerated ranges are species specific and the various factors may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another.

(b) Temperature

The temperature at which cultures are maintained should ideally be as close as possible to the temperature at which the organisms were collected; polar organisms (<10°C); temperate (10-25°C); tropical (>20°C). Most commonly cultured species of microalgae tolerate temperatures between 16 and 27°C, although this may vary with the composition of the culture medium, the species and strain cultured. An intermediate value of 18-20°C is most often employed. Temperature controlled incubators usually use constant temperature although some models permit temperature cycling. Temperatures lower than 16°C will slow down growth whereas those higher than 35°C are lethal for a number of species.

(c) Light

As for plants, light is the source of energy which drives photosynthetic reactions in algae and in this regard intensity, spectral quality and photoperiod need to be considered. Light intensity plays an important role but the requirements greatly vary with the culture depth and the density of the algal culture. At higher depths and cell concentrations, the light intensity must be increased to penetrate through the culture. Too high light intensity (e.g., direct sunlight, small container close to artificial light) may result in photoinhibition. Most often employed light intensities range between 100 and 200μEsec^{-1}m^{-2} which corresponds to about 5-10% of full daylight (2000μEsec^{-1}m^{-2}). Moreover, overheating due to both natural and artificial illumination should be avoided. Light may be natural or supplied by fluorescent tubes emitting either in the blue or the red light spectrum as these are the most active portions of the light spectrum for photosynthesis. Light intensity and quality can be manipulated with filters. Many microalgal species do not grow well under constant illumination although cultivated phytoplankton develops normally under constant illumination and hence a light/dark (LD) cycle is used (maximum 16:8 LD, usually 14:10 or 12:12).

(d) pH

The pH range for most cultured algal species is between 7 and 9 with the optimum range being 8.2-8.7, though there are species that dwell in more acid/basic environments. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished by aerating the culture. In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH which may reach limiting values of up to pH 9 during algal growth.

(e) Salinity

Marine algae are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat which is obtained by diluting sea water with tap water. Salinities of 20-24 g/l are found to be optimal.

(f) Mixing

Mixing is necessary to prevent sedimentation of the algae. It is also needed to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (e.g., in outdoor

cultures) and to improve gas exchange between the culture medium and the air. The latter is of primary importance as the air contains the carbon source for photosynthesis in the form of carbon dioxide. For very dense cultures, the CO_2 originating from the air (containing 0.03% CO_2) bubbled through the culture limits the algal growth and pure carbon dioxide may be supplemented to the air supply at a rate of 1% of the volume of air. CO_2 addition furthermore buffers the water against pH changes as a result of the CO_2/HCO_3^- balance.

Mixing of microalgal cultures may be necessary under certain circumstances: when cells must be kept in suspension in order to grow especially important for heterotrophic dinoflagellates; in concentrated cultures to prevent nutrient limitation effects due to stacking of cells and to increase gas diffusion. It should be noted that in the ocean, cells seldom experience turbulence and hence mixing should be gentle. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test-tubes, erlenmeyers), aerating (bags, tanks) or using paddle wheels and jet pumps (ponds). Not all algal species can tolerate vigorous mixing. The following methods may be used: bubbling with air (may damage cells); plankton wheel or roller table (about 1 rpm.) and gentle manual swirling. Most cultures do well without mixing, particularly when not too concentrated, but when possible, gentle manual swirling (once each day) is recommended.

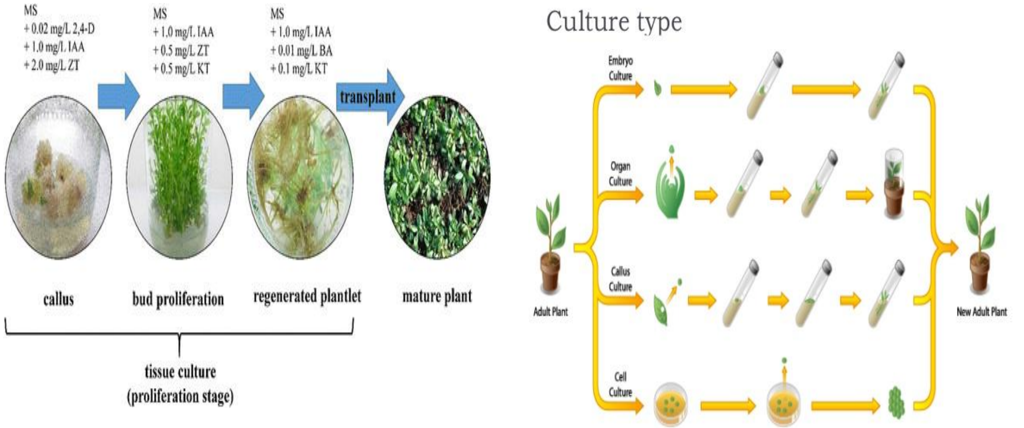


Fig 2: Flow Chart of Tissue Culture in Marine Algae

2.3.3.1 Tissue Culturing of Marine Algae

- For most cultured algal species, the pH is between 7 and 9 with the optimum range being 8.2-8.7.

- In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH which may reach limiting values of up to pH 9 during algal growth with highest growth rates of 1.5% d^{-1} around 7.5 to 8.0.

- At lower pH levels, the plants might show tip discoloration and dieback suggesting a limitation in CO_2 availability.

- Weekly cleaning to remove epiphytes is mandatory for the first 3 to 6 weeks.

- Cleaning procedures such as rinsing under tap or distilled water, brushing with a soft toothbrush under running water or using an ultrasonic bath with distilled water for 30 seconds is essential.

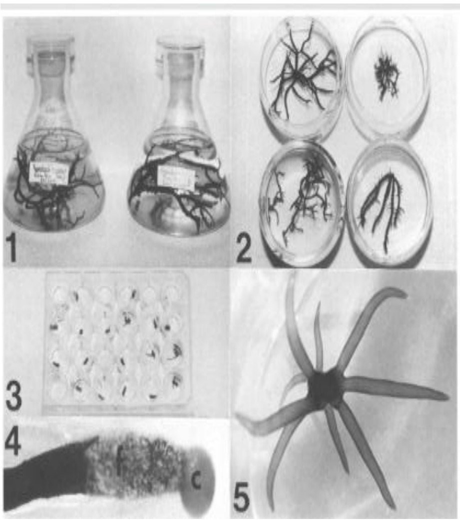


TABLE 1. Special tissue culture medium TC-1 compared with algal growth medium SWMD-1 (all amounts per 1 litre)

Compound	TC-1	SWMD-1
$NaNO_3$	1 mM	0.5 mM
$(NH_4)_2SO_4$	0.5 mM	—
NH_4NO_3	—	0.5 mM
Na_2SiO_3	—	0.2 mM (optional)
Na_2EDTA	30 μM	30 μM
$FeCl_3$	2.0 μM	2.0 μM
$MgSO_4$	2.0 mM	0.2 mM
H_3BO_3	0.1 mM	0.05 mM
$Na_2MoO_4 \cdot H_2O$	0.1 mM	—
Inorganic micronutrients (provided from a stock solution of which 2 ml gave the following)		
$MnCl_2$	7×10^{-3} mM	
$ZnCl_2$	8×10^{-5} mM	
$CoCl_2$	2×10^{-5} mM	
$CuCl_2 \cdot H_2O$	2×10^{-7} mM	
Organic micronutrients (provided from a stock solution, 0.3 μm Millipore filter-sterilized; 2 ml stock in 1 litre gave the following)		
Thiamine–HCl	0.5 mg	
Nicotinic acid	0.1 mg	
Ca–pantothenate	0.1 mg	
Biotin	1.0 μg	
Folic acid	2.0 μg	
Thymine	5.0 μg	
Cobalamine	1.0 μg	
Kinetin	1 mM	
NAA	1 mM	
TRIS	—	5 mM
Liver extract	—	10 mg
Filtered seawater[a]	—	1000 ml
Sterile diluted seawater[b] to make	1000 ml	—
pH[c]	7.3	7.5

NOTE: Millipore filtration (0.3 μm) was used for sterilization of the liquid medium.
[a]Salinity approximately 30‰.
[b]Seawater : double distilled water = 4:1, salinity approximately 24‰.
[c]pH adjusted with 1 N HCl.

Fig 3: Tissue Culture Media in Marine Algae

2.3.3.2 Culture Methods

Algae can be produced according to a great variety of methods from closely controlled laboratory methods to less predictable methods in outdoor tanks. Indoor culture allows control over illumination, temperature, nutrient level, contamination with predators and competing algae whereas outdoor algal systems though cheaper make it very difficult to grow specific algal cultures for extended periods. Open cultures such as uncovered ponds and tanks of indoors or outdoors are more readily contaminated than closed culture vessels such as tubes, flasks, carboys, bags etc. Axenic cultivation can be also chosen by using algal cultures free of any foreign organisms such as bacteria, but this cultivation is expensive and difficult because it requires a strict

sterilization of all glassware, culture media and vessels to avoid contamination. These constraints make it impractical due to expensiveness for commercial operations. On the other hand, non-axenic cultivation though cheaper and less laborious is more prone to crash, less predictable and often of inconsistent quality.

Different types of algal cultures are used worldwide. The most routinely adopted include batch, continuous and semicontinuous ponds and photobioreactors.

2.3.4 Somatic Hybridization

Algae are the most heterogeneous assemblage of autotrophic primitive plants and belong to the division Thallophyta. They are much diversified in form, function and structure and grow in almost all habitats. Most algal production is contributed to the detrital food chain either as particulate or dissolved organic matter and a small part is directly consumed by grazing. In the marine environment, algae form 90% of the total marine flora and thus playa very vital role in sustaining the productivity of the oceans. Among the algae in marine habitats, the microscopic, free-floating or swimming forms are known as phytoplankton and the macroscopic, benthic forms are called seaweeds. Seaweeds generally grow abundantly on rocky substrata in intertidal and subtidal regions where adequate light for growth penetrates. Ever since the utilization of these resources for different purposes, several workers have investigated the possibilities of using these resources more effectively for the production of economically and commercially important products world-wide.

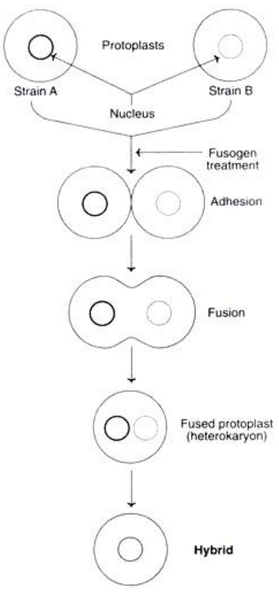


Fig 4: Diagrammatic Representation of Protoplast Fusion in Somatic Hybridisation

Very few studies have been made on somatic hybridization of algae in general and seaweeds in particular. Primke *et al.*, (1978) were the first to successfully demonstrate the possibility of protoplast fusion in a coenocytic siphonaceous marine green alga, *Acetabularia*by bringing cells into contact with each other mechanically. Subsequently, Ohiwa (1978) studied the behaviour of cultured fusion products of freshwater filamentous, *Zygnema* and *Spirogyra* (green algae) protoplasts using polyethylene glycol (PEG) with high pH. Later, Matagne *et al.*, (1979) reported nuclear fusion of cell wall mutants of the unicellular green alga, *Chlamydomonas* following fusion by the PEG method. Subsequent works involved concentrated efforts towards fusion of economically important seaweeds. As in some unicellular freshwater algae, cell fusion and subsequent growth of fusion products of *Viva linza* and *Monostroma angicava* protoplasts have been reported by Zhang (1983). In an ambitious attempt, Saga *et al.*, (1986) accomplished interphyllum fusions between *Enteromorpha* and *Porphyra* protoplasts using both PEG and electro fusion methods but without achieving success in regenerating fusion products. Later, however, Fujita and Migita (1987) reported for the first time subsequent regeneration of new plants from fusion products of *Porphyra*. Since then, similar studies reporting success in growing interspecific heteroplasmic fusion products to adult plants were described in Somatic Hybridization in Algae 485 *Ulva* (Reddy and Fujita, 1989), *Porphyra* (Fujita and Saito, 1990) and *Gracilaria* (Cheney, 1990). With a different approach, Kapraun (1989, 1990) described karyogamy and regeneration of hybrids from intraspecific fusion products of zoospores of *Enteromorpha* and *Ulvaria*.

2.3.4.1 Somatic Hybridization: Aspects, Applications and Limitations

The conventional method to improve the characteristics of cultivated plants for years has been sexual hybridization. The major limitation of sexual hybridization is that it can be performed within a plant species or very closely related species. This restricts the improvements that can be done in plants.

The species barriers for plant improvement encountered in sexual hybridization can be overcome by somatic cell fusion that can form a viable hybrid. Somatic hybridization broadly involves in vitro fusion of isolated protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant.

Plant protoplasts are of immense utility in somatic plant cell genetic manipulations and improvement of crops. Thus, protoplasts provide a novel opportunity to create cells with new genetic

constitution. Protoplast fusion is a wonderful approach to overcome sexual incompatibility between different species of plants.

2.3.4.2 Aspects of Somatic Hybridization

i) Fusion of Protoplasts
ii) Selection of Hybrid Cells
iii) Identification of Hybrid Plants

(A) Fusion of Protoplasts

As the isolated protoplasts are devoid of cell walls their in vitro fusion becomes relatively easy. There are no barriers of incompatibility at interspecific, inter-generic or even at inter-kingdom levels for the protoplast fusion. Protoplast fusion that involves mixing of protoplasts of two different genomes can be achieved by spontaneous, mechanical or induced fusion methods.

(i) Spontaneous Fusion

Cell fusion is a natural process as is observed in case of egg fertilization. During the course of enzymatic degradation of cell walls, some of the adjoining protoplasts may fuse to form homokaryocytes (homokaryons). These fused cells may sometimes contain high number of nuclei ranging from 2-40.This is mainly because of expansion and subsequent coalescence of plasmodermal connections between cells. The frequency of homokaryon formation was found to be high in protoplasts isolated from dividing cultured cells. Spontaneously fused protoplasts, however, cannot regenerate into whole plants except undergoing a few cell divisions.

(ii) Mechanical Fusion

The protoplasts can be pushed together mechanically to fuse. Protoplasts of *Lilium*and *Trillium* in enzyme solutions can be fused by gentle trapping in a depression slide. Mechanical fusion may damage protoplasts by causing injuries.

(iii) Induced Fusion

Freshly isolated protoplasts can be fused by induction. There are several fusion inducing agents which are collectively referred to as fusogens e.g. $NaNO_3$, high pH/Ca^{2+}, polyethylene glycol, polyvinyl alcohol, lysozyme, concavalin A, dextran, dextran sulfate, fatty acids and esters, electro fusion. Some of the fusogens and their use in induced fusion are described.

Treatment with Sodium Nitrate

The isolated protoplasts are exposed to a mixture of 5.5% $NaNO_3$ in 10% sucrose solution. Incubation is carried out for 5 minutes at 35°C followed by centrifugation (200xg for 5 min). The protoplast pellet is kept in a water bath at 30°C for about 30 minutes during which period the protoplast fusion occurs. $NaNO_3$ treatment results in a low frequency of heterokaryon formation particularly when mesophyll protoplasts are fused.

High pH and High Ca^{2+} Ion Treatment

This method was first used for the fusion of tobacco protoplasts and is now in use for other plants also. The method consists of incubating protoplasts in a solution of 0.4 M mannitol containing 0.05 M $CaCl_2$ at pH 10.5 (glycine-NaOH buffer) and temperature 37°C for 30-40 minutes. The protoplasts form aggregates and fusion usually occurs within 10 minutes. By this method, 20-50% of the protoplasts are involved in fusion.

Polyethylene Glycol (PEG) Treatment

This has become the method of choice due to its high success rate for the fusion of protoplasts from many plant species. The isolated protoplasts in culture medium (1 ml) are mixed with equal volume (1 ml) of 28-56% PEG (mol. wt. 1500-6000 Daltons) in a tube. PEG enhances fusion of protoplasts in several species. This tube is shaken and then allowed to settle. The settled protoplasts are washed several times with culture medium.

Advantages

- It results in a reproducible high-frequency of heterokaryon formation
- It renders low toxicity to cells.
- It results in reduced formation of bi-nucleate heterokaryons.
- It is non-specific and therefore can be used for a wide range of plants.

Electro-Fusion

In this method, electrical field is used for protoplast fusion. When the protoplasts are placed in a culture vessel fitted with microelectrodes and an electrical shock is applied, protoplasts are induced to fuse. This technique is simple, quick and efficient and hence

preferred by many workers. Moreover the cells formed due to electro-fusion do not show cytotoxic responses as is the case with the use of fusogens including PEG. The major limitation of this method is the requirement of specialized and costly equipment.

Mechanism of Fusion

The fusion of protoplasts involves three phases - agglutination, plasma membrane fusion and formation of heterokaryons.

i) Agglutination (Adhesion)

When two protoplasts are in close contact with each other, adhesion occurs. Agglutination can be induced by fusogens e.g. PEG, high pH and high Ca^{2+}.

ii) Plasma Membrane Fusion

Protoplast membranes get fused at localized sites at the points of adhesion. This leads to the formation of cytoplasmic bridges between protoplasts. The plasma membrane fusion can be increased by high pH and high Ca^{2+}, high temperature and PEC.

- High pH and high Ca^{2+} ions neutralize the surface charges on the protoplasts. This allows closer contact and membrane fusion between agglutinated protoplasts.
- High temperature helps in the intermingling of lipid molecules of agglutinated protoplast membranes so that membrane fusion occurs.
- PEG causes rapid agglutination and formation of clumps of protoplasts. This results in the formation of tight adhesions of membranes and consequently their fusion.
- Formation of Heterokaryons

The fused protoplasts get rounded as a result of cytoplasmic bridges leading to the formation of spherical homokaryon or heterokaryon.

(B) Selection of Hybrid Cells

About 20-25% of the protoplasts are actually involved in the fusion. After the fusion process, the protoplast population consists of a heterogenous mixture of unfused chloroplasts, homokaryons and heterokaryons. It is therefore necessary to select the hybrid cells, heterokaryons. The commonly used methods employed for the selection of hybrid cells are biochemical, visual and cytometric methods.

NOTES

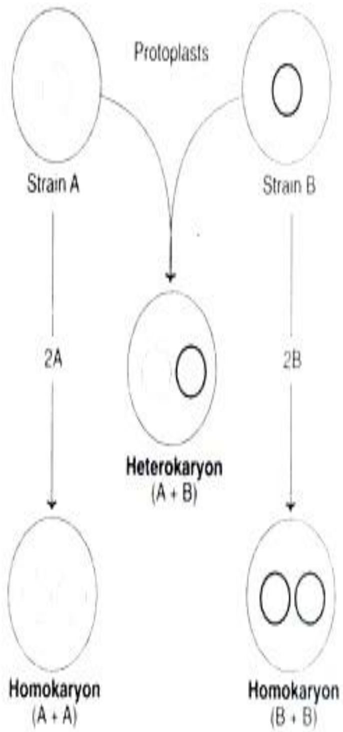


Fig 5: Fusion Product in Protoplast

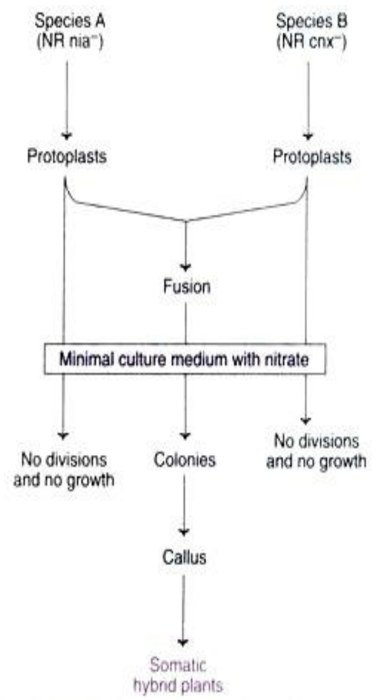


Fig 6: Isolation of Somatic Hybrid Cells

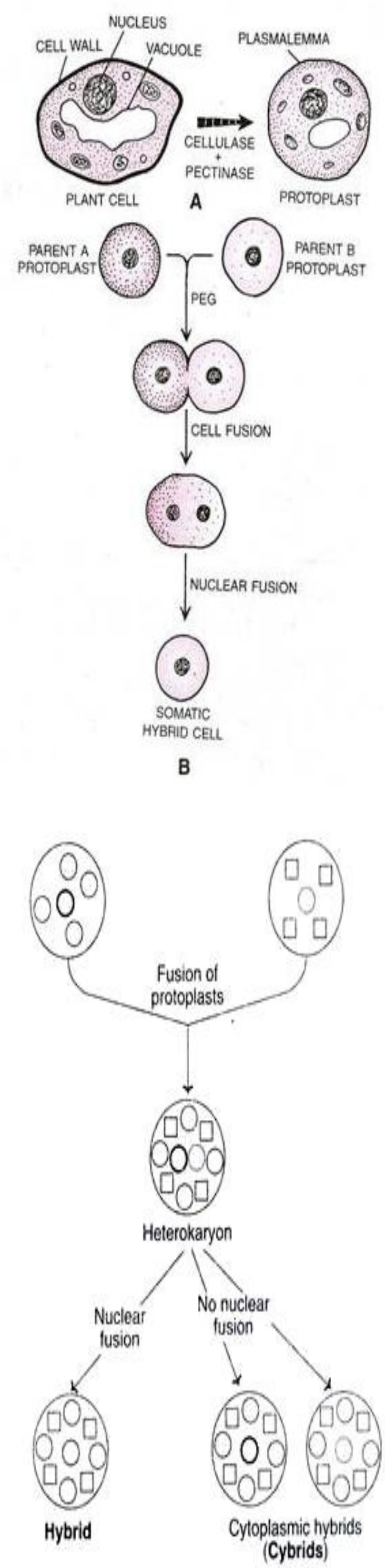


Fig 7: Diagrammatic Representation of Hybrid and Cybrid Cells

2.3.5 Biotechnological Importance of Algae

NOTES

- Algae as renewable energy source:*Chlorella*, *Dunaliella*, *Gracillaria* and *Sargassum* produce fuels like diesel, gasoline, methane, butanol, ethanol and aviation fuel.
- Algae as food: *Alaria*, *Laminaria*, *Sargassum*, *Porphyra* is popular as food in Japan and Europe.
- They can grow on land or water (arid/saline/alkaline/marshy) which is unsuitable for crop cultivation.
- They scavenge green house gases and can be used for carbon dioxide mitigation.
- Algae are cheap source for waste water treatment and biogas production.
- Genetically engineered algae are used to enhance biofuel production and as source of protein and vitamin rich food and fodder.
- Algae are used as biofertilizer for crops as rich source of nitrogen, phosphorous, potassium, iodine, iron, calcium, silica and vitamins.
- Algae have been recommended for pesticide and heavy metal bioremediation.
- Algae are used in formation of biosolar cells.
- Algal storage materials like starch, gelatin and lipids are used as gelling agents in jellies, ice-creams, confectioneries and bacteriological media.
- Algae have therapeutic importance: Chlorellin from *Chlorella* is a broad spectrum antibiotic.
- Algal pigments have antioxidant properties and therefore used in the formulation of age proofing cosmetics.

2.3.6 Algal Genomics

Algae genetic engineering is not prevalent owing to vector desig n issues (i.e. plasmids or viruses) that can be effectively integrated into t he algae, adopted by the cell and expressed in a satisfactory manner. It is abundantly evident that the development of gene editing techniques is a possibly strong force for good in terms of both human and animal health and addressing the difficulties that we are still facing in an increasing worldwide population. This includes the development of approaches to modify microalgae strains in order to improve productivity, robustness, harvest ability and process potential. Genetically modified algae comprise for the most part a couple of strains with most research on the green microalga, *Chlamydomonas reinhardtii*, a laboratory model.

Algae carry three separate genomes where nuclear genome contains the vast majority of genetic material and coding potential. The red macroalga, *Grateloupia taiwanensis* carries the most gene-rich organelle genome and contains 233 protein-coding genes. The red microalga, *Cyanidioschyzon merolae* possess least gene-rich nuclear genome encodes 4,775 proteins. Because of the larger size of nuclear genomes and their propensity for repetitive regions which makes assembly of sequencing reads more difficult we have access to fewer nuclear genomes compared with plastid and mitochondrial genomes.

Overall, to date, about 20 distinct species of microalgae including *Cyanobacteria* have been genetically modified effectively. It is only lately that there has been any advance in algae gene editing. A team at Cellectis in France using TALENs accomplished the first effective implementation of a gene editing strategy in a microalga in the marine diatom, *Phaeodactylum tricornutum*. More recently, in *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica*, CRISPR / Cas9 and CRISPR / Cpf1 have been effectively recorded.

2.3.6.1 Genetic Engineering of Algae

Current microalgae genetic engineering research focuses grea tly towards industrial and environmental applications. Several approach es have been developed to improvise the production and generation of microalgae, lipids and the capture of CO_2 efficacy.

(a) Engineering of Microalgae for Carbohydrate and Protein

As proteins are one of the main biochemical's produced in algae, the engineering of algae for enhanced protein production is of at most important. Since proteins are made from amino acids, the kinetics of amino acid synthesis are under study to be adjusted by providing changes in the expression levels of the corresponding enzymes involved. This in turn can be achieved by changing the related gene characteristics. However, when synthetic protein expression increases too high, related pathway gets suppressed and thereby production decreases. This has been trespassed by knocking out of relevant gene. However this arise several toxic production on the host cellular growth.

(b) Engineering Microalgae for Lipid Storage

Microalgae normally do not produce large amount of lipid but under stress condition like lack of nitrogen, microalgae produce energy

storage products like starch and lipid. This property has been utilised for production of genetically modified algae for lipid production. But as lipid production increases, the rate of cell division reduces. This problem is bypassed by providing inducible promoters like copper-responsive elements as inducible promoters in *C. reinhardtii* and nitrate-responsive species of diatoms are taken as inducible promoters. Another approach to increase lipid accumulation is by decreasing lipid catabolism. For this process, knocking out of essential enzymes like acyl-CoA oxidase, acyl CoA synthase, fatty acyl CoA dehydrogenase and carnitineacyl transferase I are under study which in turn are involved in β oxidation.

(c) Engineering of Microalgae for Photosynthetic Efficiency

Microalgae are great models for enhancing photosynthetic efficiency. Microalgae absorb more light than required for their photosynthetic necessity.

$$CO_2 + H_2O + 9.5hv \rightarrow 1/6\ C_6H_{12}O_6 + O_2$$

From the equation it is evident that an increase in photon utilization property in microalgae is directly proportional to the ability of its CO_2 fixation and also in light conversion efficiency. Enhanced photosynthetic efficiency has been achieved through reducing the size of the chlorophyll antenna that in turn is necessary and important in absorbing light. This is achieved by inducing mutagenesis through keeping microalgae for a long period in high intensity. However, once when the cells are exposed to low intensity light, the cells will get reverted to normal stage. But this approach permits increased light penetration on high density cultures and also allows a maximum rate of photosynthesis since cells are subjected to photo inhibition since less light is absorbed by their light harvesting complexes.

Another approach in reducing chlorophyll antennae is by changing nutritional requirements and levels through microscale, a respective gene alteration and exploitation, however this has gain less attention. Even though random mutagenesis strategies has been endorsed to generate mutants with less or small chlorophyll antennae, recent works in *C. reinhardtii* reported with efficient use of an RNAi-based strategy to knock down both LHCI and LHCII.

2.3.6.2 Construction of Transformation and Expression Vectors

Temporal permeabilisation generation by various means enabling entry of DNA to cell is the basis of algal transformation methods. Once entered, cytoplasm entry to nucleus and genome does not need any external application of force. Integration happens through intigimate recombination process inside nucleus. Even though it is not difficult to permeabilise cell, but difficulties arise when host cell fail to recover the pressure and damage created by permeabilisation. Several methods are employed for generation of transformed algal cells. The most popular among them are described in this unit.

(a) Micro Projectile Bombardment

It is also referred as gene gun transformation, micro particle bombardment or biolistic method of generation of viable algal cells. This method includes DNA coated heavy metal like gold that allows easy transformation of cells even organelles. Coated heavy metals are called microprojectiles. This has been successfully applied on *Chlamydomonas reinhardtii*. This biolistic procedure appears to be the most efficient way of introducing DNA into the chloroplast genome. This is probably because the chloroplast occupies over half of the volume of the cell providing the microprojectile with a large target. Electroporation has been shown to be the most efficient way of introducing DNA into the nuclear genome with maximum transformation frequencies of two orders of magnitude higher than obtained using glass bead method. This has also been applied on *Volvox carteri*, *Dunaliella salina*, *Laminaria japonica* etc.

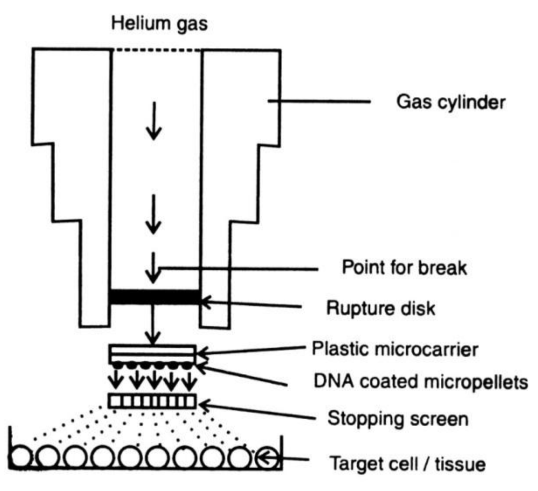


Figure: 8 Micro Projectile Bombardment

(b) Protoplast Agitation

This is less expensive and easy method of transformation in viable algal cells. This procedure involves creation of a suspension of micro algae agitated together with macro or microparticle, polyethylene glycol and DNA. Examples for micro particle include silicon carbide. Whiskers in turn is a compound of silicon and carbon approximately 0.3 - 0.6 µm thick and 5 -15 µm long. This method is highly applicable for cells with rigid cell wall like *Chlamydomona sreinhardtii* and also in less rigid cells. In *Chlorella ellipsoidea* which lacks cell wall, agitation is carried out with protoplast and DNA alone without the need of microparticle.

(c) Electroporation

This method is employed in naked cells, cell wall reduced mutants cells, protoplast and cells with thin cells in which the designed electrodes passes voltage higher than the dielectric strength. The created electric pulse disturbs plasma membrane phospholipid layer and allows DNA to enter into cell. Eg. *Cyanidioschyzon merolae*, *Dunaliella salina*.

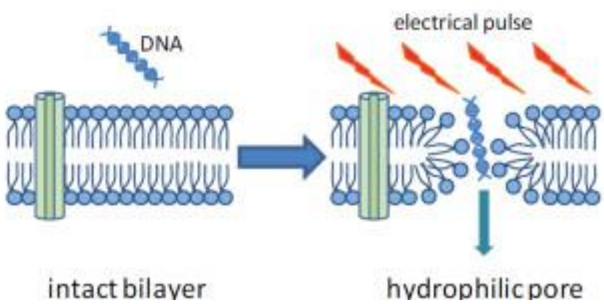


Fig 9: Electroporation

(d) *Agrobacterium tumefaciens* Mediated Transformation

Tumour inducing Ti plasmids of *Agrobacterium* integrate into host genome and transfer DNA. This has been exploited in algal cells by infecting algae with *Agrobacterium tumefacians*. Astonishingly, algae does not develop tumour. This has been tried in two algal species such as in multicellular red algae, *Porphyra yezoensis* and in unicellular green algae, *Chlamydomonas reinhardtii*.

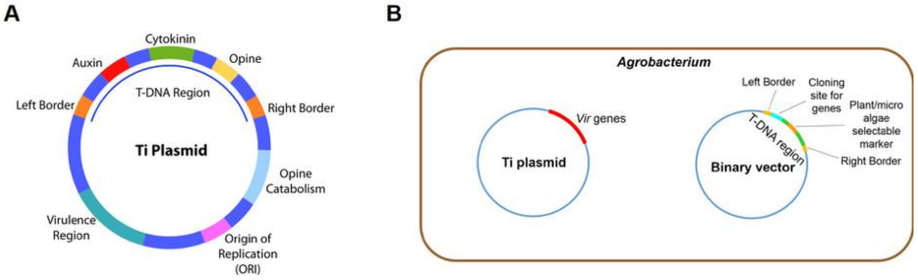


Figure 10: Agrobacterium Mediated Microalgae Transformation

Expression Vectors

(a) Algevir - Viral Based Vector for Microalgae

An inducible geminiviral vector leading to Rep-mediated replication of the expression cassette allows the production of antigenic proteins at high levels. This system called Algevir allows the production of complex viral proteins (from *Zaire ebolavirus*) and bacterial toxin subunits (B subunit of the heat-labile *Escherichia coli* enterotoxin) which retains their antigenic activity. The highest achieved yield is 1.25 mg/g fresh biomass (6 mg/L of culture) which will attain 3 days after transformation. The Algevir system allows for a fast and efficient production of recombinant proteins overcoming the difficulties imposed by the low yields and unstable expression patterns frequently observed in stably transformed microalgae at the nuclear level; as well as the toxicity of some target proteins.

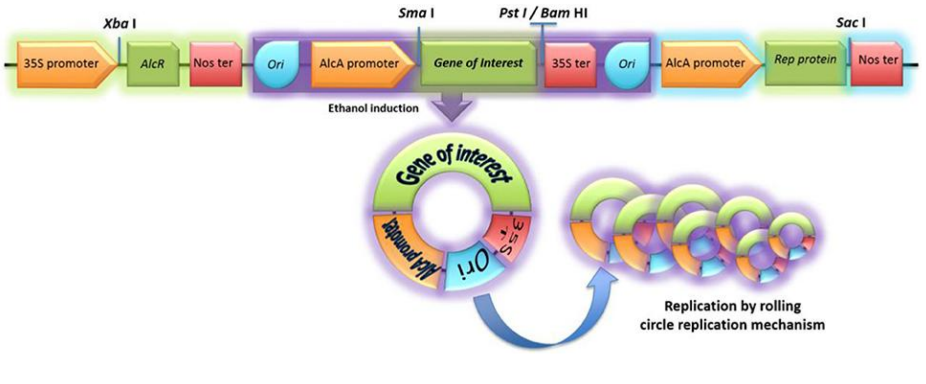


Fig 11: Algevir Vector design for Microalgae

(b) Plasmid Based Expression System

The expression vector pBI221 and adAGUS constructed using polyA signal and 35S promoter from pRT101and aadA from Puc-atpx-aadA.T .plasmid pBI221aadAGUS carries the GUS gene as a reporter gene and aadAas as the selectable gene. Introduction of antibiotic resistance into *Chlamydomonas* cells was achieved by the transformation of the cells with the bacterial aadA gene to induce expression of aminogly- coside 3‘‘-adenyl transferase (AAD) that gives the transformants resistance to spectinomycin and streptomycin. Plasmid pBI221aadAGUS with its promoters and terminators could stably be integrated with the native DNA of *Chlamydomonas reinhardtii* by biolistic bombardment with heterologous genes and the level of expression is higher.

2.3.6.3 Methods of Gene Introduction

A range of methods have been implicated to edit algal genome. Like use of transgenes as a means of stable expression of CRISPR/Cas and on other hand by means of electroporation for introducing preassembled active ribonucleoprotein (RNP: enzyme + guideRNA) complexes further more by a hybrid approach in which Cas9 produced from a transgene and rest of editing particulars are delivered to the nucleus.

Through transgene method, transgenes are used to initially deliver the CRISPR reagents. After edition, transgenes are not necessary and transgene-free, genome-engineered progenies can be generated by mating or breeding as they are located elsewhere in the genome. But CRISPR/Cas9 or CRISPR/Cpf1 RNPs direct delivery bypasses transgenesis. Desired changes in a genome are made possible without introducing a foreign gene through RNA complex method. This is made effected by introducing an active protein-RNA complex into the cell, further targeting the expected alterations or changes to the nuclear genome. The active RNPs exhibit a half-life of around 24-48 h, thus neglecting or reducing the effect of any off-target mutations which are nonexistent in plants. Thus, RNPs can be considered as safe and efficient molecules for gene introduction without off target.

Types of Modification	Change to Genome
Transgene	Stably integrated foreign gene
Cisgene	Stably integrated gene: from same species or closely related

Artificial Chromosome	Stably inherited artificial chromosome
Transgene driven change (stable or transient)	Subtle mutation to native genes. Stable transgene-driven = conventional transgenic; transient transgene: (DNA) or RNA-driven = no integrated transgene.
Ribonucleoprotein (RNP)-driven change	Precise mutation to native genes only

Table 1: Types of Gene Introduction Methods

2.4 CHECK YOUR PROGRESS QUESTIONS

1. Methods of Gene introduction in Algae
2. Microprojectile Bombardment
3. Somatic Hybridization
4. Tissue Culture Application in Algae
5. Cell Fusion
6. Algevir

2.5 ANSWERS TO CHECK YOUR PROGRESS

1. Transgene, Cisgene, Artificial chromosome, Transgene driven change, Ribonucleoprotein (RNP)-driven change.
2. This technique is also known as particle bombardment, particle gun method, biolistic process, Microprojectile bombardment or particle acceleration.
3. Somatic hybridization is a technique which allows the manipulation of cellular genomes by protoplast fusion.
4. Tissue culture is the growth of tissues or cells in an artificial medium separate from the organism
5. Cell fusion is an important cellular process in which several uninuclear cells (cells with a single nucleus) combine to form a multinuclear cell known as a syncytium.
6. Viral based vector for microalgae transformation process.

2.6 SUMMARY

- ✓ Our ability to culture microalgae has advanced rapidly over the past 50 years. We now have sufficient understanding of their physiological requirements to culture hundreds probably thousands, of different species.
- ✓ The fundamental techniques used to culture algal cells are reviewed in this section. Use of these relatively simple

techniques has led to an improved understanding of the factors that limit algal growth.

- ✓ There are many compounds of commercial potential in microalgae and finding economically viable methods of producing them will challenge future generations.
- ✓ They are also the main source of some organic molecules important in human nutrition and physiology such as long chain polyunsaturated "omega three fatty acids.
- ✓ Development of gene editing techniques is a possibly strong force for good in terms of both human and animal health
- ✓ Algal genetic engineering is a promising field in biotechnology with various applications in the area of generation of GMOs for lipid protein and carbondioxide production and consumption.
- ✓ Various transformation techniques are available for algal systems that permit the recovery of viable transformants.
- ✓ A number of methods like transgene and cis gene and RNAi induced gene modification and introductions methods are employed for generation of successful genetically modified algae in macroscale.

2.7 KEYWORDS

Algal Biotechnology, Tissue Culture, Cell fusion, Somatic Hybridization, Protoplast Fusion. Culture Media, Algal genomics, Gene editing in algae, construction of vector.

2.8 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Write a brief note on Algal Biotechnology.
2. Applications of Algal Biotechnology.
3. Comment on Cell Fusion.
4. Tissue Culture.
5. Describe the general Scope of Algal Biotechnology.
6. Give a brief account on vectors employed in Algal Transformation.

Long Answer Questions

1. Elaborate on Somatic Hybridization.
2. Mention the applications of Algal Biotechnology.
3. Narrate Tissue Culture in suitable Picture.
4. In detail explain the Scope of Algal Biotechnology in Industries.
5. Illustrate the Cell Fusion with a suitable diagram.

NOTES

6. What are the various methods of transformation of DNA in microalgae?

2.9 FURTHER READINGS

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UNIT - III BIOTECHNOLOGICAL APPLICATIONS OF ALGAE

Structure

3.1 INTRODUCTION

Algae are a class of photosynthetic organisms found in both marine and freshwaters habitats. As these organisms have a short doubling time, they are considered among fastest growing creatures. In few years, a focus has been shifted towards these organisms due to their food and fuel production capability. The term "algae" encompasses a variety of organisms found throughout the world in or near waterbodies. Algae species are estimated to number in the tens of thousands. Though most algae are photosynthetic or autotrophic, some are heterotrophic deriving energy from the uptake of organic carbon such as cellulosic material.

Because algae are naturally able to replicate rapidly and produce oils, proteins, alcohols and biomass they have attracted the attention of researchers and industrial producers seeking alternatives to oil. Algae thrive on organic carbon or CO_2 and nutrients such as nitrogen and phosphorus. Growth conditions and the availability of

sunlight, carbon and nutrients affect the metabolism of algae of whether they produce lipids or carbohydrates. However, manipulation of nutrients has not proved successful in increasing algal productivity. Researchers, for instance have found that when algae naturally produces hydrocarbons, the molecules can readily substitute for today's petroleum uses with limited growth and reproduction.

Biotechnology is broadly defined as any technique that uses live organisms viz., bacteria, viruses, fungi, yeast, animal cells and plant cells to make or modify a product, to improve plants or animals or to engineer microorganisms for specific uses. It encompasses genetic engineering, inclusive of enzyme and protein engineering, plant and animal tissue culture technology, biosensors for biological monitoring, bioprocess and fermentation technology. Biotechnology is essentially and interdisciplinary are consisting of biochemistry, molecular chemistry, molecular and microbiology, genetics and immunology etc. it is concerned with upgradation of quality and also utilization of livestock and resources for the well-being of both plants and animals.

Modern biotechnology holds considerable promise to meet challenges in agricultural production. It makes use of life sciences, chemical sciences and engineering sciences in achieving and improving the technological applications of the capabilities of the living organism of their derivatives to make products of value to man and society. It is used in living systems to develop commercial processes and products which also include the techniques of Recombinant DNA, gene transfer, embryo manipulation, plant regeneration, cell culture, monoclonal antibodies and bio-processed engineering. These techniques can transform ideas into practical applications. Certain crops can be genetically altered to increase their tolerance to certain herbicides. Biotechnology can be used to develop safer vaccines against viral and bacterial diseases. It also offers new ideas and techniques applicable to agriculture and also develops a better understanding of living systems of our environment and ourselves. It has a tremendous potential for improving crop production, animal agriculture and bioprocessing.

New approaches in biotechnology can develop high yielding and more nutritious crop varieties, improve resistance to disease and also reduce the need for fertilizer and other expensive agricultural chemicals. It could also improve forestry and its products, fibre crops and chemical feed-stocks. Plant biotechnology play a key role in the massive production of improved crop varieties through in vitro tissue culture followed by clonal propagation as well as in their genetic improvement. They can also help in propagating plant species which contain useful and biologically active substances Eg food additive,

pigment, pharmaceuticals, biopesticides etc. Organ tissue and cell culture could be more efficient than conventional extraction.

Biotechnology helps to isolate the gene, study its function and regulation, modify the gene and reintroduce it into its natural host of another organism. It helps unlocking the secrets of diseases resistance, regulates growth and development or manipulates communication among cells and among other organisms. It is a comparatively new technique and is used in the field of agriculture and horticulture. This mainly involves manipulation in the genetic code including processes like gene transfer, tissue culture, monoclonal antibody preparation and protoplast fusion. These processes help in increasing yield, producing better quality products both in plants and animals, increasing resistance to pests and herbicides, micro propagation in several crops etc.

Biotechnology research goals therefore include finding ways to increase the reproductive rate, improve metabolism of inputs and enhance the production of desired oils, fuel-grade alcohols or proteins in useful species. Researchers have found that many algae species are adaptable to genetic engineering, expressing complex proteins and accumulating recombinant proteins to very high levels. Algae are being used from a long period of time due to their high biomass production rate in different extreme habitats as compared to cereal based crops. A wide range of metabolites from algae containing various bioactive compounds are yet to be exploited. Algae mainly contain over 50% starch which can be converted into ethanol. The cell walls of algae are mainly composed of different carbohydrates due to which they can be used as a raw material in similar manner as in cellulosic ethanol. By the process of pyrolysis, algal biomass can be converted into organic liquids, acetic acid, acetone and methanol, various clean and cost effective gaseous products. With passage of time, new technologies are being developed for cultivation of algae over a large scale all over year, under different climatic zones varying from tropical to moderate climate.

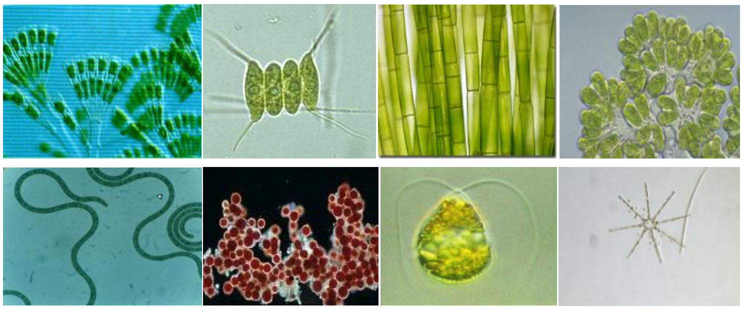


Fig 1: Phenotypical diversity of Microalgae based on their genetic and environmental characteristics.

Microalgae are an extremely heterogeneous group of organisms described as a life-form not a systematic unit. They are regarded as unicellular photoautotrophic (contain chlorophyll a) microorganisms, than can be eukaryotic or prokaryotic. The diversity of the microalgae is very broad and is reflected in an equally wide range of metabolisms and biochemical properties as a diversity of pigments, photosynthetic storage products, cell walls and mucilages, fatty acids and lipids, oils, sterols, hydrocarbons and bioactive compounds including secondary metabolites. This biodiversity implies that groups of organisms are differentiated by some measure of the extent to which their gene pools are separated and how this is expressed phenotypically. The phylogeny of algae and related organisms has evolved dramatically in recent years. Molecular and ultrastructural evidence of evolutionarily conserved features (e.g. ribosomal RNA gene sequencing, flagellar hairs and roots, plastid and mitochondrial structure, the mitotic apparatus) has combined to create an exciting, dynamic field of inquiry.

3.2 OBJECTIVES

This unit provides the reader with the information regarding the biotechnological applications of algae in agriculture and environment. This includes various applications viz., nitrogen fixation, biodiesel, biogas, bioethanol, bio-oil, bio-hydrogen, fertilizers and many more.

3.3 ALGAE AND ITS BIOTECHNOLOGICAL APPLICATIONS

Algae are being used from a long period of time due to their high biomass production rate in different extreme habitats as compared to cereal based crops. Algae are categorized as third generation biofuels due to their many advantages over different agricultural based crops. The concept of using algae for energy production is not new. Till now the cost of production of algae biofuels is very high due to its limited cultivation systems. But with passage of time, new technologies are being developed for cultivation of algae over a large scale all over year, under different climatic zones varying from tropical to moderate climate. Algae have a potential to fix atmospheric carbon rich gases due to which they act as quenchers of carbon dioxide and nitrogen oxides released from different sources. About 1 kg of algae biomass is capable of fixing approximately 1.8 kg of carbon dioxide. Algae can use wastewater containing high amount of nitrogen and phosphorus for their growth with the benefits of providing biofuels and also helping to get rid from excess nitrogen and phosphorus.

A wide range of metabolites from algae containing various bioactive compounds are yet to be exploited. *Haematococcus pluvialis*, freshwater algae is a source for producing commercially available

astaxanthin pigment, whereas *Chlorella vulgaris* as a food supplement and algae *Dunaliella* species for β-carotene production. Marine biomass could also be used as a feedstock to produce different fuels as bioelectricity by co-firing, bioethanol, biodiesel, bio-oil by pyrolysis and biomethane via fermentation. The market potential of algal biofuels is vast due to their sustainable technology to replace fossil fuels. Algae mainly contain over 50% starch which can be converted into ethanol. The cell walls of algae are mainly composed of different carbohydrates due to which they can be used as a raw material in similar manner as in cellulosic ethanol. By the process of pyrolysis, algal biomass can be converted into organic liquids, acetic acid, acetone and methanol, various clean and cost effective gaseous products. The different criteria for algae to fit as a candidate for bioenergy production includes: high biomass productivity, easy harvesting by mechanical techniques, cost effective production than other biomass present.

In present era, the cultivation cost of algal biomass is very high for production of biofuels only. Therefore, the need of hour is to compensate the cost of biofuel production from other byproducts that can be produced from algae. Currently various outputs from agriculture and food produced by different sustainable strategies are in market. The production of various co-products along with biofuels will make the process more feasible, cheap and expected to provide new opportunities due to its positive effect on sustainability.

3.3.1 High-Value Natural Products in Microalgae

Microalgae are the source of several forms of high-value compounds such as carotenoids, polyunsaturated fatty acids (PUFAs), proteins, antioxidants and pigments. Characterized by high protein and nutrient contents, some species such as *Arthrospira platensis* (a Cyanobacterium also known as *Spirulina platensis*) and *Chlorella vulgaris* (a green alga) are used as feed, food additives and diet supplements. In other cases, specific high-value compounds are isolated from appropriate strains. Relatively few proteins have been purified from microalgae for commercial use, but *Spirulina* is a rich source of phycocyanin, a protein that constitutes 14% of the dry weight of this Cyanobacterium. The US Food and Drug Administration (FDA) have approved phycocyanin from *Spirulina* as a blue food colourant. Moreover, phycocyanobilin, the tetra pyrrole chromophore of phycocyanin, manifests fluorescent properties that have been exploited for labelling of antibodies in immunofluorescence and flow cytometry. In mammalian tissues, it can be enzymatically reduced to phycocyanorubin (a close homologue of bilirubin) and inhibits the activity of NADPH oxidase, thus reducing the generation of reactive oxygen species. It has been suggested that a regular intake of phycocyanobilin may provide protection against cancer and other

diseases. Moreover, recent studies have proven the beneficial health effect of microalgae (*Chlorella*, *Spirulina*) by increasing natural killer cell levels and stimulating immune and anti-inflammatory system response in humans.

Carotenoids are important products that are extracted from microalgae and indeed the first commercialized product derived from algae was β-carotene. It is produced in very high amounts by *Dunaliella salina*, a halophilic alga growing in saline habitats which makes the cultures less susceptible to contamination. What differentiates *Dunaliella* β-carotene from the synthetic product (present only in the form of all-trans isomer) is that it is rich in the 9-cis isomer and a negative effect of the use of all-trans isomer, such an effect on plasma cholesterol levels and atherogenesis has been reported from mice studies. Another example of a carotenoid with a well-established and growing market in the nutraceutical area is astaxanthin from the freshwater green alga, *Haematococcus pluvialis*. Astaxanthin is mainly used as a feed supplement and pigmentation source for *Salmon* and *Shrimp* farming, but due to its high antioxidant properties (10-fold greater than other carotenoids) and protective activities it also finds many applications in the pharmaceutical and cosmetic industries. Astaxanthin has also been shown to prevent bacterial infection, vascular failure and cancer.

Fatty acids are the other natural components produced commercially from microalgae. Several marine algal species are rich in omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA; e.g. from *Isochrysis* strain T-iso and *Pavlovalutheri*), eicosapentaenoic acid (EPA; e.g. *Nannochloropsis gaditana*, *Nannochloropsis oculata*), and alpha-linolenic acid (e.g. *Rhodomonas salina*, *Tetraselmisuecica*). Oils from *Nannochloropsis*, *Rhodomonas* and *Tetraselmis* have higher antioxidant properties than fish oils partly because of a high content of valuable carotenoids (fucoxanthin, lutein, neoxanthin, alloxantin) and polyphenols. It is a known fact that intensive fishing endangers many fish species, algal oils may provide an alternative to fish oils in diets in the future.

Other algal species such as *D. salina* and *Botryococcus braunii* can accumulate up to 60% of storage lipids as triacylglycerides (TAGs), potentially making them a valuable source of oil for biodiesel production. Compared with plants, algae exhibit higher productivities and theoretically could give 10- 100-fold higher yields of oil per acre although such capacities have not yet been achieved on a commercial scale. Nevertheless, algae appear to be a potential solution to the controversial food vs fuel problem that is associated with the use of fertile land to produce plant-derived biofuels. TAGs are not the only way in which microalgae could be exploited for biofuels. Under

anaerobic conditions and sulphur depletion, some microalgae produce hydrogen gas which could be used as an alternative to fossil fuels in the future. An attempt had been made to produce hydrogen from *Chlamydomonas reinhardtii* using natural sunlight. Although successful, the overall yields did not exceed those obtained in lab-scale settings and so this technology is at an early stage.

While the above compounds are well-established microalgal products with known potential, other microalgae are being studied for new compounds with useful properties. Some Cyanobacteria have a poor reputation in the popular press for causing toxic blooms because they are able to produce hepatotoxins and neurotoxins (e.g. anatoxin, jamaicadine, L-beta-N-methylamino-L-alanine). However, research on Cyanobacteria is undergoing a renaissance because some identified metabolites and their derivatives have been shown to have potential as next generation antiviral, anticancer and antibacterial drugs. Several of these drugs have even successfully reached phase II and III clinical trials. A large number of promising natural compounds are derived from filamentous marine genera such as *Lyngbya*, *Symploca* and *Oscillatoria*. Usually they are short peptides built from non-canonical amino acids by the hybrid polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS).

Some of the most promising candidate anticancer agents are derivatives of *dolastatin* 10, a peptide originally isolated from Sea hare, *Dolabellaauricularia*. The first one, TZT-1027 (*soblidotin*) is a microtubule polymerization inhibitor that exhibits antitumour activity in preclinical models, manifesting stronger activity than paclitaxel. Another analogue of *dolastatin* 10 (*monomethylauristatin E*) linked to an antibody is already approved by the FDA and used in therapies for patients with Hodgkin lymphoma.

3.3.2 Biotechnological Applications in Agriculture and Environment

3.3.2.1 Improvement of Soil Fertility

Some Cyanobacteria are able to reduce atmospheric nitrogen to ammonia, a process where oxygen evolved by photosynthetic activity in the same cell is detrimental to nitrogen fixation. Strategies to avoid oxygen range from temporal separation of nitrogen fixation and oxygen evolution (in many unicellular and filamentous, non heterocysts strains) to spatial separation and cellular differentiation into nitrogen fixing heterocysts (in filamentous cyanobacteria). Heterocysts are terminally differentiated cells whose interior becomes anaerobic, mainly as a consequence of respiration allowing the oxygen-sensitive process of nitrogen fixation to continue.

The regulation of dinitrogen fixation has been extensively studied in the heterocyst system.

Diazotrophic cyanobacteria require sunlight as a sole energy source for the fixation of carbon and nitrogen. Therefore, they have great potential as biofertilizers and their use will decrease fuel demand for fertilizer production. The agronomic potential of heterocystous cyanobacteria either free-living or in symbiotic association with water fern, *Azolla* has long been recognized. This had led to the development of small scale biotechnology involving the use of paddy soils with appropriate Cyanobacterial strains as biofertilizers in rice fields as has been reported in China, Egypt, Philippines and India. Cyanobacteria are congenial biofertilizers for rice based cropping systems, being the major components of wetland rice ecosystems which are easily available and serve as the cheapest sources of natural biofertilizers. Whereas the incorporation of genes into rice plants by using tissue culture and modern genetic tools remains as an ambitious research goal, the use of even long term alternative to synthetic nitrogen fertilizers particularly in developing countries and the world as a whole. However, one of the weaknesses in the technology is the heavy application of several toxic agrochemicals especially herbicides which are reported in most cases as inhibitors of Cyanobacterial diazotrophic growth and in some cases as mutagenic. Therefore, a successful biotechnology requires the selection of suitable diazotrophic strains as biofertilizers that could tolerate the field-dose concentrations of herbicides.

3.3.3 Phytopathogen Biocontrol

3.3.3.1 Applications of Chemical Pesticides

Soil is a dynamic system in which the physical, chemical and biotic components are in a state of equilibrium. Application of insecticides without considering the other soil constituents disturbs this equilibrium which adversely affects the productivity of the soil. Maintenance of the soil biota other than the harmful pests helps in better crop nutrient management and maintenance of soil health. Insecticides frequently exert inhibitory or stimulatory effects on the growth or other activities of microorganisms either in pure culture or in the field. Blue-green algae especially the nitrogen-fixers Cyanobacteria represent the major microorganisms which contribute soil fertility. These organisms play an important role in this system by providing a steady input of fixed nitrogen. Most of the soil and aquatic microscopic algae are sensitive to insecticides due to the fact that algae are engaged in photosynthesis and that many insecticides interfere with the process. Until now many pesticides from different chemical and artificial sources were used as acaricidal, fungicidal and insecticide agents. These pesticides affect the distribution of fauna in their natural

ecosystems. Also, the unjustifiable and unsafe applications of these pesticides on soils and plants cause accumulation of different undesirable chemicals in the crop which may be a bigger indirect factor in human diseases.

3.3.3.2 Biological Control

In the last decades, different researchers have studied the replacement of chemical pesticides by natural components of different plant and microalgal sources as insecticide agents, acaricide agents and fungicidal agents. These natural materials in addition to their lethal activities on pests, preserves the environment of pollution, maintain the equal distribution of fauna and also to keep the beneficial animals. Fungi and bacteria are the main biological agents that have been studied for the control of plant pathogens particularly soil-borne fungi. Cyanobacteria have received little attention as potential biocontrol agents of plant diseases. Different concentrations of dilute aqueous extracts from nitrogen-fixing Cyanobacterium, *Nostoc muscorum* were reported to be efficient in the control of a damping off. Also, it was found that the growth of the plant pathogens, *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, damping-off causal agents was inhibited by extracts from cells of *N. muscorum*or by extracellular products of this Cyanobacterium. With additional research, it should be possible to develop thin film formulations (polymers) of bactericidal and fungicidal Cyanobacterial products that would confer protection against soil-borne pathogens that attack seeds and seedlings when applied to high volume seeds and remain competitive. One of the modern and advanced biotechnological research is with usage of different algal taxa of different habitats (marine, fresh and soil) as a biological control for many animal or plant diseases and also against agricultural pests. Some of these researchers studied the antimicrobial activities against some human pathogenic bacteria, fungi and toxic micro-algae. Research is also conducted on the study of toxic effects of some algal metabolites against insects. For example, it was found that a free floating unicellular *Chlorella elliposidea* produces some substances which affect the development and immature stages of mosquitoes. Some Cyanobacteria and green algae produce substances that inhibited larval development and delayed the survival and development of the adult female of mosquitoes. The toxicity of *Microcystis aeruginosa* against the larvae of *Culex pipiens* increased gradually during the first days of exponential phase to the maximum and then gradually decreased at the beginning of the stationary phase. There is much evidence that the production of extracellular substances by blue-green algae is widespread and sometimes quantitatively important.

3.3.3.3 Exopolysaccharide

Cyanobacteria produce extracellular polymers of diverse chemical composition, especially exopolysaccharides that enhance microbial growth and as consequence, improve soil structure and exoenzyme activity. Maintenance of adequate levels of soil organic matter is essential for a sustainable and high production of crops. Cultivation alters the structural stability of soil and reduces the amount of N and soil organic matter. The nature of this labile organic matter is not fully known, but a major portion of it could be microbial biomass.

Incorporation of organic materials in soil promotes microbial growth and enzymatic activity in the soil. Some cyanobacteria excrete slime or mucilage that becomes dispersed around the organism and to an extentpartially dissolves in the culture medium or in the soil solution. One way to positively affect nutrient content and soil structure is to add Cyanobacteria. Application of algal biofertilizers is also useful for the reclamation of marginal soils such as saline-alkali and calcareous soils. *N. muscorum* can improve the aggregate stability of a saline soil, where the increase in soil aggregation is mainly due to exopolysaccharide secreted by microorganisms or exopolysaccharide added to soil after death and cellular lysis. Cyanobacteria can be incorporated into soil as organic matter and also as a source of enzymes as they produce acid and alkaline extracellular phosphatases that are active in solution or located in the periplasmatic space of the cell wall. Both biomass and exopolysaccharides incorporated into soil induce a growth promotion of other microorganisms and increased the activity of soil enzymes that participate in the liberation of nutrients required by plants.

3.3.3.4 Removal of Heavy Metals

Algae biomass can be used as an inexpensive biomaterial for the passive removal of toxic heavy metals. The relative affinity of raw *Sargassum* biomass for various divalent metal cations was determined at environmentally relevant concentrations to be Cu >Ca> Cd >Zn > Fe. Also microalgae have been used to remove heavy metals from wastewater.

The uptake of Cd (II) and Pb (II) by microalgae in the presence of colloidal organic matter from wastewater treatment plant effluents was studied. The uptake of Cd by *Chlorella kessleri* was consistent with the speciation and measured free metal ion concentrations while Pb uptake was much greater than that expected from the speciation measurement.

Brown algae have proven to be the most effective and promising substrates due to the biochemical composition of the cell wall which contains alginate and fucoidan. *Ascophyllum nodosum* (brown algae) has proven to be the most effective algal species to remove metals of cadmium, nickel and zinc from monometallic solutions compared to green and red algae. A similar study proved that brown algae, *Fucus vesiculosus* gave the highest removal efficiency of chromium (III) at high initial metal concentrations. Another algal species, *S. obliquus* was examined for degrading cyanide from mining process wastewater. It was observed that cyanide was degraded up to 90% after introduction of algae into the system. *Spirogyra condensata* and *Rhizoclonium hieroglyphicum* also employed as biosorption substrates to remove chromium from tannery wastewater.

3.3.4 Nitrogen Fixation

Algae have different pathways to fix atmospheric carbon dioxide and to efficiently utilize the nutrients to convert it into biomass. Algae have a potential to fix atmospheric carbon rich gases due to which they act as quenchers of carbon dioxide and nitrogen oxides released from different sources. About 1 kg of algae biomass is capable to fix approximately 1.8 kg of carbon dioxide. Algae can use wastewater containing high amount of nitrogen and phosphorus for their growth with benefits of providing biofuels and also helping to get rid from excess nitrogen and phosphorus.

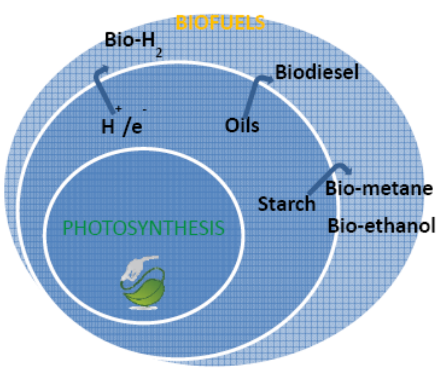


Fig 2: The Role of Algae Photosynthesis in Biofuels Production

3.3.5 Different Algae Based Fuels

In modern era, fossil fuel depletion and global warming has led to the world's eyes on production of bioenergy from algal biomass. Therefore the key plans to reduce poverty are increased access and energy security. Currently, the only alternative to replace the fossil fuel

consumption and dependency is the production of biofuels from algal biomass.

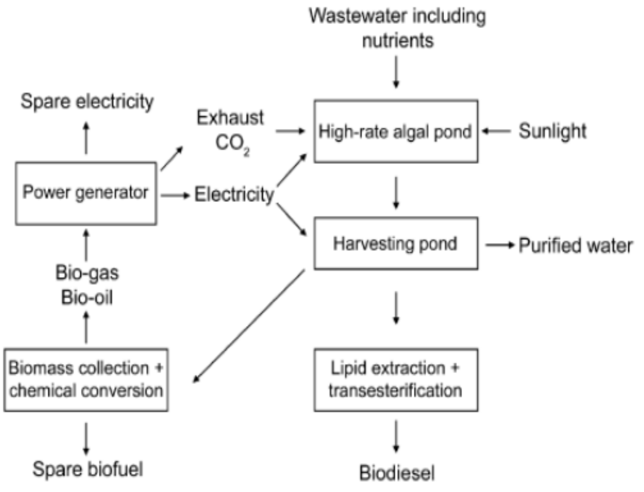


Fig 3: A Flow diagram showing how wastewater resources could be used for Sustainable Algal-based Biofuel Production

BioFuels

The term biofuel refers to any type of solid, liquid or gaseous fuels which can be derived from renewable raw materials. The key points for any conversion process are form and quantity of biomass, kind of energy and economic return from the product. Agricultural crops are classified as first generation biofuels due to their use for food or feed but there is always a race between food and fuel to produce enough biofuel to contribute a large portion towards total fuel consumption. In comparison to biofuels produced from agricultural feedstock, algae cultivation does not occupy agricultural land to compete with agriculture. With the combined benefits of large biomass productivity, wastewater treatment, all year production, chemical composition of algae, oil content of algae can be controlled by change of algae cultivation techniques. In fuel industry algae biofuels have been emerged as a clean, nature friendly, cost effective solution to other fuels. Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae as a food have been explored for different applications as in production of single cell proteins, pigments, bioactive substances, pharmaceuticals and cosmetics. Algae are categorized as third generation biofuels due to their many advantages over different agricultural based crops. The concept of using algae for energy production is not new. Till now the cost of production of algae biofuels is very high due to its limited cultivation systems.

Bio-Oil

Bio-oils are produced by a process called thermo-chemical conversion which in absence of oxygen converts biomass into oil along with char and gas at very high temperature. Bio-oils are quite similar to petroleum oils due to which they can be used as a substitute. The bio-oil formation process is categorized into two steps: Pyrolysis and Thermo-chemical liquefaction. Pyrolysis is performed at a very high temperature (350-530°C) for production of a liquid, a gaseous and solid part. The liquid part is made up of an aqueous and a non-aqueous phase called bio-oil or tar and biomass is dried. During thermo-chemical liquefaction, wet biomass is hold upon lower temperature and high pressure of about 300°C and 10 MPa respectively. The bio-oil contains various organic compounds accumulated as lipids, proteins, carbohydrates in algae and as compared to lipids present in algae and amount of yield is high. A number of microalgae have been investigated to produce bio-oil via pyrolysis or thermal liquefaction. For example, the formation of different hydrocarbons by pyrolysis of *Dunaliella* sp. biomass. Bio-oil yields from microalgae have been reported up to 41% for *Spirulina*, about 24%-45% for microalgae, *Scenedesmus*, about 37% for *Dunaliella* and up to 49% for *Desmodesmus*. Bio-oil yield from macroalgal biomass has been reported up to 23% after liquefaction process while in macrolagal biomass *Laminaria saccharina* it accounts for 63% energy restoration and *Laminaria saccharina* yielded 79% oil after hydrothermal liquefaction. The freshwater macroalgae, *Oedogonium* and *Cladophora* yielded only 26% and 20% respectively.

Biodiesel

In recent years the study of biodiesel has been widely recognized and it is mostly produced from oil seed crops like soybean oil, palm oil and rapeseed oil. The production cost of biodiesel mainly depends upon type of raw material used as it is a critical factor accounting for 50-85 % of total fuel price. For production of cost effective biodiesel, assessment of feedstock is important in terms of productivity, quality and exploitation of by-products which should be taken into consideration. The process of conversion of raw material lipids mainly triacyleglycerols/free fatty acids into non-toxic and eco-friendly biodiesel is called trans-esterification. The crude algal oil having high viscosity is converted into low molecular weight compounds in the form of fatty acid alkyl esters. During the process of trans-esterification, crude oil in presence of a catalyst reacts with an alcohol usually methanol and fatty acid methyl esters (FAME) are formed as final product along with glycerol. The application of acid catalyst has been regarded as advantageous, but alkali catalyst is used commercially due to its fast nature up to 400 times as compared to acid catalyst.

Chlorella vulgaris and *Chlorella protothecoides* are two main species containing high oil content and have been studied by many workers for the production of biodiesel. The potential of *Chlorella protothecoides* for Biodiesel Production Microwave-Assisted Trans-esterification was evaluated. Trans-esterification was performed with *Chlorella protothecoides* oil methanol and potassium hydroxide as the catalyst. Methanol:oil ratio, reaction time and catalyst:oil ratio are investigated as process parameters affected methyl ester yield. 9:1 methanol/oil molar ratio, 1.5% KOH catalyst/oil ratio and 10 min were optimum conditions for production of highest biodiesel yield. Microalgal biodiesel is mainly composed of unsaturated fatty acids. The algal biomass from wastewater contains a mixture of various algae and hence different fatty acid profiles can be obtained.

Biohydrogen

The diversity of biofuel sources has become an urgent energy issue. In current years, much attention has been paid towards the bio-hydrogen production. But still, production of bio-hydrogen on large scale is not feasible due to costly process and low biomass concentration. In some studies it has been reported that exposing some algae species to various environmental stress like depriving light in algae could trigger appreciable amount of hydrogen gas. But this technology is still in its beginning stage and process can be developed or improved till higher level. To overview the current scenario of hydrogen production from algae the work of many researchers may be referred. The three pathways suggested for the production of hydrogen are: 1. Direct Photolysis 2. Indirect Photolysis 3. ATP driven pathway. Direct Photolysis is made feasible only when the produced hydrogen and oxygen are continuously removed. In this process, photosynthesis and water splitting are linked leading to the production of hydrogen and oxygen together which results in bigger security risks also increasing the cost for separation of hydrogen and oxygen. Furthermore, the hydrogenase enzyme used in the process is very oxygen sensitive. Due to these reasons, indirect processes are favoured mostly. Under anaerobic and sulphur limited environment, starch contained in cell walls of algae converted into hydrogen up to some extent. In most of the studies it has been found that Cyanobacteria are the main producers of bio-hydrogen through biological approach. Hydrogenase and nitrogenase enzymes act as catalyst in this process.

Biogas

Organic material like crop biomass or liquid manure can be used to produce biogas via anaerobic digestion and fermentation. Mixtures of bacteria are used to hydrolyze and break down the organic biopolymers

(i.e. carbohydrates, lipids and proteins) into monomers which are then converted into a methane-rich gas via fermentation (typically 50-75% CH_4). Carbon dioxide is the second main component found in biogas (approximately 25-50%) and like other interfering impurities has to be removed before the methane is used for electricity generation. Microalgae biomass is a source of a vast array of components that can be anaerobically digested to produce biogas. The use of this conversion technology eliminates several of the key obstacles that are responsible for the current high costs associated with algal biofuel including drying, extraction and fuel conversion and as such may be a cost-effective methodology. Several studies have been carried out that demonstrate the potential of this approach. A recent study indicated that biogas production levels of 180 mL/g.d of biogas can be realized using a two-stage anaerobic digestion process with different strains of algae with a methane concentration of 65%. Calorific value is directly correlated with the microalgae lipid content and under nitrogen starvation results in a significant increase in the caloric value of the biomass with a decrease of the protein content and a reduction in the growth rate. The energetic cost of biomass harvesting and lipid recovery is probably higher than the recovery energy especially since most of the techniques involve biomass drying. When the cell lipid content does not exceed 40%, anaerobic digestion of the whole biomass appears to be the optimal strategy on an energy balance basis, for the energetic recovery of cell biomass. Anaerobic digestion well explored in the past will probably re-emerge in the coming years either as a mandatory step to support large scale microalgae cultures or as a standalone bioenergy producing process. This technology could be very effective for situations like integrated wastewater treatment where algae are grown under uncontrolled conditions using strains which are not optimized for lipid production.

Biomethane Production

In these days, worldwide production of biogas from biomass is gaining importance. An anaerobic digester contains synergistic microbial populations which convert algal organic compounds (lipid, protein, carbohydrate) to methane and carbon dioxide. Methane is widely used both as a fuel and chemical feedstock. The potential of different feedstocks such as algae, wood, grass, solid waste for biomethane production and found algae biomass as potential candidate for production of biomethane was investigated. The biomass productivity of algae is generally higher than land plants, but its growth is influenced by limiting of different nutrients. The thermal pre-treatment of microalgae for enhancement of biomethane production was examined by using *Chlorella* sp. Methane yields from untreated algae were 155 mL g^{-1}VSadd, while thermal pre-treatment at 70°C and 90°C for 0.5 h increased the methane yield by 37% and 48% respectively.

Thermal pre-treatment at 121°C for 0.3 h resulted in the highest methane yield (322 mL g^{-1}VSadd) which was 108% higher than the untreated algae.

Bioethanol

Bioethanol production from algae has gained unusual importance due to its high biomass productivity, diversity, variable chemical composition, high photosynthetic rates of these organisms. Algae are the optimal source for production of bioethanol due to large amount of carbohydrates/polysaccharides and thin cellulose walls. Commonly two processes are used for production of bioethanol from algae, 1) Fermentation and 2) Gasification. In various countries, commercial ethanol is produced from sugary and starchy crops on a large scale by fermentation. The biomass is grinded and the starch is converted to sugars by different means. The starch of microalgae is released from the cells with the aid of enzyme, acid, alkali and yeast, *Saccharomyces cerevisiae* is added to initiate fermentation and sugar is converted to ethanol. The final product from fermentation (ethanol) is then drained and pumped to holding tank attached to a distillation unit. Ueno *et al*., (1998) has examined the potential of marine green alga, *Chlorococcum littorale* to produce ethanol under dark fermentation and about 27% of starch contained in algal cells was used within 24 hours at 25ºC. The feedstock potential of algal biomass was assessed to produce bioethanol and to encourage its use as renewable biofuel for providing tenable option. Examples of green algae employed for bioethanol are: *Dunaliella*, *Chlorella*, *Chlamydomonas*, *Arthrospira*, *Sargassum*, *Spirulina*, *Gracilaria*, *Prymnesium parvum*, *Euglena gracilis* and *Scenedesmus*. The optimization of bioethanol process was done by Plackett-Burman experimental design followed by immobilization technique on supported solid materials. The results of the experiment showed that the sugar concentration, pH level and the inoculums size have a significant effect on the bioethanol production by *S. cerevisiae* with conversion efficiency of 47.1% while immobilized yeast showed conversion efficiency of 52%.

The feasibility of producing bioethanol from brown seaweeds, *Ascophylum nodosum* and *Laminaria digitata* was pre-treated and hydrolyzed by using dilute sulphuric acid and commercially available enzymes. Large amount of fermentable sugars were obtained with glucose and rhamnose being the predominant sugars respectively. Fermentation of the resultant seaweed sugars was performed using two non-conventional yeast strains: *Scheffersomyces* (Pichia) *stipitis* and *Kluyveromyces marxianu*. Although the yields of ethanol were quite low at around 6 g/L, macroalgal ethanol production was slightly higher using *K. marxianus* compared with *S. stipites*.

Acid pre-treatment can release up to 49% of sugars from algal biomass, whereas enzymatic hydrolysis can release up to only 20% of sugars based on its dry weight. For *Ulva lactuca*, ethanol yields was reduced even after release of sugars after hydrothermal pre-treatment, but the yield was enhanced by the mechanical pressing up to 0.14 gg^{-1}dry matter due to removal of inhibitors formed during fermentation. Among all algae (brown, green, red algae) which can be converted into ethanol by fermentation, brown algae are considered as a potential feedstock for ethanol production due to high carbohydrate content and ease of cultivation. *Laminaria hyperborean* contains Laminarin and mannitol which underwent fermentation to produce large amount of ethanol by yeast, *Pichia angophorae*. The fermentation of red algae has also been reported using acid hydrolysis. The algae were converted into sugars, but ethanol yield was low as up to 45% theoretical yield maximum. The fermentation of seaweed produces ethanol yield between 0.08 and 0.12 kg^{-1} dry seaweed, depending upon the genera of algae and different methods for pre-treatment and hydrolysis. Studies revealed that the ethanol yield of a dry sea weed was found up to 0.281 gg^{-1} with 80% efficiency higher than previous experimental yields. Another study proposed an ethanol yield of 0.296 gg^{-1} dry seaweed for commercial purpose. Still, a conversion efficiency of 50% for ethanol from seaweed is a challenging task and has not been seen at scale. A current investigation has achieved a potential ethanol yield of approximately 90 litres $tone^{-1}$ of dried macroalgae, but this is low in comparison to land based crops for biofuel production. In Brazil, bioethanol is commercially used as ethanol or mixture of ethanol and petroleum in 86% of sold cars. But still, there are many limitations of bioethanol as low vapour pressure, low energy density and low flame luminosity.

Biobutanol Production

In Asia, Europe and South America, algae cultivation is mainly accomplished for bioethanol and biogas production, whereas in USA, algae are gaining attention for biobutanol production. Since 100 years, butanol has been used as a fuel in transportation and has been recommended as a potential candidate for biofuel, not only to improvise, but to take place of ethanol as petroleum additive due to its low vapour pressure and high energy density. The bacteria used in butanol production not only digest starch and sugars but also utilize cellulose present in algal biomass; therefore butanol production could be as economic as ethanol. By anaerobic fermentation, many *Clostridium* sp. are capable of yielding acetone, butanol and ethanol (ABE) by utilizing both hexoses and pentoses sugars by a process called as ABE fermentation, but limitation of butanol is inhibition of fermentation leading to less yield and productivity. Butanol has been produced from fermentation of algae, *Ulvalactuca* by *Clostridium*

strains, but butanol yield is lower up to 0.16 g butanol g^{-1} than ethanol yield under similar condition. *Ulvalactuca* used as a feedstock for fermentation by *Clostridium* was pre-treated with hot water followed by enzymatic hydrolysis by commercial cellulases for production of acetone, butanol and ethanol (ABE) with a yield of 0.35 g ABE g^{-1} sugar. However these results determined the potential of seaweed, *Ulva lactuca* as a feedstock for ABE fermentation, but this was projected for consecutive production of 1, 2 propanediol (propylene glycol) in a seaweed biorefinery as a replacement to fossil fuel derived products rather than just a root for butanol as fuel. Macrolagae, *Ulva* extracted from Jamaica Bay, New York City was used for production of butanol on a pilot scale from saccharification of sugars. In a study on Brown algae, *Saccharina* butanol fermentation from acid treated organic matter was determined but the yields were low as up to 0.12 g g^{-1} extracted soluble solids. The main fermentation products derived from laminarin were mannitol and glucose but a major portion of alginates was obstinate and did not undergo fermentation process. Butanol fermentation from microalgae-derived carbohydrates was studied after ionic liquid extraction to compare between ionic liquid extracted algae (ILEA) and hexane extracted algae (HEA) for acetone, butanol and ethanol (ABE) fermentation. Direct ABE fermentation of ILEA and HEA showed a butanol titre of 4.99 and 6.63 g L (-1) respectively with a feasibility of producing biodiesel and butanol from a single feedstock for reducing the feedstock cost of each process.

3.3.6 Fertilizers

Both macro and microalgae contain numerous compounds to promote germination, leaf or stem growth, flowering and can also be used as a biological protectants agent against plant diseases and are used in various coastal areas. After the recovery of oil and carbohydrates from macroalgae and microalgae, still many nutrients are left in spent biomass. One potential application for this leftover biomass is to use as a biofertilizer and will increase economic potential of algae for reuse in cultivation after extraction of nutrients. The left-over biomass will be used as fertilizer. Most of the Cyanobacteria are able to fix atmospheric nitrogen and can be used as effective biofertilizers. They play a major role in conserving and building soil fertility for increasing rice growth and yield as natural biofertilizer. After water, nitrogen is second important factor for growth of plants in fields and its requirement met by fertilizers. With the help of Blue green algae (BGA), various physical, chemical properties of soil are improved along with increasing yield and saving of fertilizer nitrogen. Blue green algae like *Nostoc*, *Anabaena* and *Tolypothrix* are capable of fixing atmospheric nitrogen and are used as inoculants for growing paddy crops in both upland and low land conditions.

3.3.7 Processed Food Ingredients

The products like agar, alginates and carrageenans are among most valuable products that can be derived from algae due to their gelling and thickening properties. In past years, a considerable growth has been observed in area of algae research and development in fields like protoplast fusion, macroalgal cell cultures and transgenic algae.

Agar

Agar derived from macroalgae has many applications in food products such as frozen food, deserts, candies, fruit juices along with industrial uses like paper sizing, textile printing, and molecular biology as agarose and in various biomedical fields to produce capsules, tablets, anticoagulants.

Carrageenans

Carrageenan derived from algae are water soluble polysaccharides and most commonly used than agar for application as emulsifying and stabilizing agents in various foods. κ- and ι-carrageenans are commonly used in various foods like jellies, jams, deserts, meat products due to their thickening action. Various pharmaceutical applications of carrageenans like antiviral, antitumor and anticoagulant have also been investigated.

Alginate

Alginate, a brown alga derived compound is used in textile industry for sizing cotton yarn and is of high importance due to its gelling properties. Alginate has chelating property and ability to make highly viscous solution which makes it a potential candidate for food and pharmacy sectors. Aquaculture Feed   Presently, various algae feeds are used for culture of various fish like larvae, juvenile fish and finfish. The most commonly used algae for aquaculture feed are *Chlorella*, *Tetraselmis*, *Pavlova*, *Phaeodactylum*, *Nanno chloropsis*, *Skeletonema* and *Thalassiosira*. By using *Chlorella* and*Spirulina*, or their mixture many companies are making aquaculture feed. *Hypnea cervicornis* and *Cryptonemia crenulata* microalgae, rich in protein were tested as shrimp diet. Hundreds of microalgae have been examined as food over last few decades, but still a less than twenty have gained importance in context of aquaculture.

3.3.8 Other Eco-friendly Applications

Other Major Environmental Applications of Algae are:

- Algae can be used for trapping fertilizers present in farm runoff. After harvesting they can be used as fertilizer.
- Since algae can grow in harsh conditions and do not require many nutrients they are cultivated in places that are not suitable for agricultural purposes therefore, they do not compete for arable land as well as use wastewater, not freshwater.
- Unlike row crops, algae do not depend on specific seasons. Algae can thrive wherever there are warm temperatures and plenty of sunlight.
- Algae are grown in seawater as well as in desert ponds. Algae can also grow in waste water and water containing phosphates, nitrates and other contaminants.
- Since algae are carbon neutral, it can help the environment by taking CO_2 from the air. Algae farms can be located near carbon producing refineries or power plants.

3.3.20 Other Applications of Algae

Algae are used in a number of other applications that are enumerated below:

- Algae can be cultivated for hydrogen generation. In 1939, German researcher, Hans Griffon discovered that the algae, *Chlamydomonas reinhardtii* could be switched from oxygen to hydrogen production.
- Algae are a complete protein containing important amino acids that are involved in metabolic processes such as enzyme and energy production. It has large amounts of complex and simple carbohydrates that provide the body with an additional fuel source. Specifically speaking, sulphated complex carbohydrates improve the regulatory response of the immune system. It also contains Omega 3 and Omega 6.
- *Chondrus crispus* or "carrageen" is a good stabilizer in milk products and reacts with casein, a milk protein those alginates in lotions and can be absorbed through the skin.
- Can be used as soil conditioners, fertilizers and as a livestock feed source. Since a number of species are microscopic and aquatic they are placed in ponds or clear tanks and harvested for the treatment of effluents pumped through ponds.
- Algae can also be used as sugar. Algae produce natural pigments that can be used instead of colouring agents and chemical dyes.

- Currently manufactured paper products are not easily recycled due to the chemical inks used. According to recyclers, algae inks are very easy to break down.
- The food sector is also attempting to replace the colouring agents presently used with colouring obtained from algal pigments.
- Algae can be used for medicine manufacture, sewage treatment and cosmetic manufacture.
- A green algae species grown in water tanks and exposed to sunlight and heat becomes bright red in color. This can be harvested and used as a natural pigment for foods such as Salmon.

3.4 CHECK YOUR PROGRESS QUESTIONS

1. How biofuels are produced from algae?
2. Algae in Agriculture - Discuss.
3. List the different uses of algae.
4. Name the algaecommonly used as a fertilizer.
5. Mention the economic and ecological importance of algae.

3.5 ANSWERS TO CHECK YOUR PROGRESS

1. In order to minimize costs, algal biofuel production usually relies on photoautotrophic culture that uses sunlight as a free source of light. Phototrophic microalgae require light, carbon dioxide, water and inorganic salts to grow. The culture temperature should be between 15 and 30°C (~60-80°F) for optimal growth.

2. Algae are a large and diverse group of microorganisms that can carry out photosynthesis since they capture energy from sunlight. Algae play an important role in agriculture where they are used as biofertilizer and soil stabilizers. Egg-laying rate in hen is also increased by algae feed additives.

3. Algae are commercially cultivated for pharmaceuticals, nutraceuticals, cosmetics and aquaculture purpose. Algae can be used to make biodiesel, bioethanol and biobutanol and by some estimates can produce vastly superior amounts of vegetable oil compared to terrestrial crops grown for the same purpose.

4. Blue green algae (Cyanobacteria) are frequently used as nitrogenous bio-fertilizer for rice crop. *Anabaena* sp., *Aulosirafertilissima* and

Nostoc muscorum are the most common species of blue green algae which are used as biofertilizers.

5. Apart from playing a vital role in the world's ecology, algae perform an important function in the world's economy. Research indicates that algae can be used by the agricultural industry in the production of fertilizers due to the high nutrient content. Algae are also used in sewage treatment to remove toxic particles.

3.6 SUMMARY

Algae and Cyanobacteria are an apparently under-explored source of natural high-value compounds and there is a rising interest in their exploitation. Just as numerous plant secondary metabolites have been used for biotechnological and biomedical purposes, there is huge scope for the identification of correspondingly valuable compounds within the vast microalgal population.

New discoveries are made regularly and new compounds and applications for industrial purposes are to be expected in the near future. Cyanobacteria and algae have the potential to be used as a bio-control agent against plant pathogenic bacteria and fungi. Few works on pesticides distributions, types, toxicity, mechanism of actions, degradations, their tolerance by the organisms and other physiological processes were reviewed and summarized. The enhanced research activity on the subject of biological control is in line with increased effort and determination by microbiologists to adapt to the conceptual scheme of integrated pest management as an acceptable ecosystem approach to disease control and to realize that biological control must become one of the basic components in pest management practices.

The amount of organic matter reduces the effect of the stability of soil aggregates. The pH and concentration of algae were concluded to have significant effect on removal of chromium thus indicating potential of algae for removal hazardous heavy metals in wastewater. The production of ethanol from brown seaweed needs higher ethanol yields for industrial process and application of all ingredients of seaweed for non-fuel based products also.

3.7 KEYWORDS

Microalgae, Cyanobacteria, Nitrogen Fixation, Biofertilizers, Carrageenans, Biofuels.

3.8 SELF ASSESSMENT QUESTIONS & EXCERCISE

Short Answer Questions

1. Define Cyanobacteria and its types
2. Explain Blue Green Algae with an example
3. What do you mean by Carotenoids?
4. Comment on the production of Azolla
5. Xenobiotics
6. Heterocyst
7. Write short notes on Seaweeds and types

Long Answer Questions

1. Explain the process of Nitrogen Fixation
2. Write notes on the biological importance of Blue-green Algae
3. Elaborate the economic and ecological aspects and importance of Algae
4. In detail explain the importance and the applications of Cyanobacteria in agriculture and environment
5. Give a brief review on the algal biofuels and their impact on agriculture and environment
6. How is Biodiesel produced from Algae?
7. Give a detailed account on the process of Somatic Fusion with schematic presentation
8. Mention about Clonal Propagation with suitable diagram

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UNIT-IV MICROBIAL PESTICIDES

Structure

4.1 INTRODUCTION

Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria and certain minerals. For example, canola oil and baking soda have pesticidal applications and are considered as biopesticides. Generally, biopesticides are made of living things, come from living things or they are found in nature. They tend to pose fewer risks than conventional chemicals. Very small quantities can be effective and they tend to break down more quickly which means it renders less pollution.

It could be generally referred that biopesticides are generally less toxic and eco-friendly. They act on non-target organisms making them desirable and sustainable tool for disease management. It plays as an important tool in pest management where pesticide resistance and environment concerns limit the use of chemical pesticide products.

4.2 OBJECTIVES

This chapter provides descriptions of biological control (biocontrol) agents of insect, disease and weed pests. It also includes the concept and practice of biological control and Integrated Pest Management (IPM).

4.3 MICROBIAL PESTICIDES

4.3.1 Bacterial Pesticides

B. thuringiensis is a widely distributed bacterium. It can be isolated from soils, litters and dead insects. It is a spore forming bacterium and produces several toxins viz., a, b, d-exotoxins and d-endotoxins which can be obtained in crystalline forms. b-exotoxin contains adenin, ribose, glucose etc. These toxins have insecticidal properties. *Bacillus thuringiensis* has been found a strong antagonist to be used as a biocontrol agent. It is an aerobic and spore forming bacterium, pathogenic to larvae of lepidoptera. After ingestation of spores, larvae are damaged as the rod-shaped bacterial cell secretes at the opposite end, a single large crystal in the cell. This crystal (toxin) is proteinaceous in nature. It gets dissolved in alkaline solution and in alkaline juice of caterpillar's digestive cavity.

4.3.2 Viral Pesticides

(a) Cytoplasmic Polyhedrosis Viruses (CPV)

Preparations of viruses or their products have been developed as effective biopesticide and being successfully used for the control of insect pests in agriculture, forestry and horticulture. This method of disease control is free from pollution, toxicity or any hazards related to plant or animal health. However, these viruses are specific and have no harmful effects on useful insect pollinators, insects yielding useful products, warm blooded animals and even man. After application viruses get entered into the mouth and digestive tract of insect pest and kill them.

(b) Nuclear Polyhedrosis Viruses (NPVs)

This belong to subgroup of Baculoviruses have been used for the preparation of potential pesticides. *Heliothis* sp. is a cosmopolitan insect pest attacking at least 30 foods and fiber yielding crop plants. They have been controlled by application of NPVs of *Baculovirus heliothis*. In 1975, Environmental Protection Agency, U.S.A. registered the *B. heliothis* preparations. At present it is marked as wettable powder under the name "Elcartm (Sandoz Inc.), 'Biotrol' VHZ (Nutrilite Products Inc.) and 'Viron/H' (International Minerals Chemical Corp.)

(c) *Lymantria dispar*

Lymantria dispar commonly known as gypsy moth is a serious pest of forest trees. It has been successfully controlled by gypsy

moth Baculovirus *i.e.* NPV preparations. NPVs product has been registered under the trade name 'Gypchek' (the Environmental Protection Agency, U.S.A) to control gypsy moth population on deciduous hardwood trees.

4.3.3 Mycopesticide

Recent studies on the use of entomopathogenic fungi for the control of insect pests have increased the interest of mycologists throughout the world. Fungal preparations are being produced commercially. Mode of action of these fungi is different from viruses and bacteria. The infective propagules (conidia, spores etc.) of the antagonistic fungi reach the haemocoel of the insect either through integument or mouth. They get attached to epicuticle, germinate and penetrate the cuticle either by germ tubes or by infection peg. They multiply in haemocoel followed by secretion of mycotoxins which result in death of insect hosts. Thereafter, mycelia spread saprophytically and grow outside the integument which later on produces conidiophores and conidia.
In England, *Verticillium lecanii* has shown as a potential antagonist against aphid pests especially for *Macrosiphonrella sanbornii, Branchycaudus helichrysi* and *Myzus persicae* affecting *Chrysanthemum* in green houses. In USSR, spraying of aphids and spider mites with *Entomophthora thaxteriana* and *E. sphaerosperma* resulted in 95% mortality within 24 hours by secreting the mycotoxins. *E. thaxteriana* suspension when applied on aphids of apple trees resulted in about 74% mortality without harmful effects on natural predators. Also, use of 1.5 kg/ha of 'Boverin' (30 x 10^9 conidia of *Beauveria bassiana* /g) with reduced dosages of chemical insecticide (Chlorophos) has been recommended in Russia for the control of Colorado beetle (Bhagat, 1991). A commercial preparation of *B.bassiana* has also been produced under the product name ‘Biocontrol FBB’ (Nutrilite Products Inc., USA).

4.4BASIC PRINCIPLE OF MICROBIAL PESTICIDE

4.4.1 Antagonism

In phytopathology, Antagonism refers to the action of any organism that suppresses or interfere the normal growth and activity of a plant pathogen such as the main parts of bacteria or fungi. These organisms can be used for pest control and are referred to as biological control agents. They may be predators, parasites, parasitoids or pathogens that attack harmful insect, weed or plant disease or any other organism in its vicinity. The inhibitory substance is highly specific in its action affecting only a specific species. Many soil microorganisms are antagonistic. They secrete a potent enzyme which

destroys other cells by digesting their cell walls and degrade the cellular material as well as release protoplasmic material which serves as a nutrient for the inhibitor organism. For example, *Aspergillus* renders an antagonistic effect on *Penicillium* and *Cladosporium*. *Trichoderma* has an effect on Actinomycetes. *Pseudomonas* exhibits antagonism on *Cladosporium*.

4.4.2 Amensalism

It is an association between organisms of two different species in which one is inhibited or destroyed and the other is unaffected. There are two basic modes: competition in which a larger or stronger organism excludes a smaller or weaker one from living space or deprives it of food and antibiosis in which one organism is unaffected but the other is damaged or killed by a chemical secretion.

A clear case of amensalism is where sheep or cattle trample grass. Whilst the presence of the grass causes negligible detrimental effects to the animal hoof, the grass suffers from being crushed. Amensalism is often used to describe strongly asymmetrical competitive interactions such as has been observed between the Spanish ibex and weevils of the genus, *Timarcha* which feed upon the same type of shrub. Whilst the presence of the weevil has almost no influence on food availability, the presence of ibex has an enormous detrimental effect on weevil numbers as they consume significant quantities of plant matter and incidentally ingest the weevils upon it.

4.4.3 Siderophores

Siderophores are the other extracellular metabolites which are secreted by bacteria (e.g.*Aerobacter aerogenes, Arthrobacter pascens, Bacillus polymyxa, Pseudomonas cepacia, P. aeruginosa, P. fluorescens, Serratia*), Actinomycetes (*Streptomyces* sp.) yeasts (*Rhodotorula* sp.), fungi (*Penicillum* sp.) and dinoflagellates (*Prorocentrum minimum*). Siderophores are commonly known as microbial iron chelating compounds because they have a very high chelating affinity for Fe^{3+} ions and very low affinity with Fe^{2+} ions. Siderophores are low molecular weight compounds. After chelating Fe^{3+} they transport it into the cells. Kloepper *et al.*, (1980) were the first to demonstrate the importance of siderophore production by PGPR in enhancement of plant growth. Siderophores after chelating Fe^{3+} make the soil Fe^{3+}deficient for other microorganisms. Consequently growth of other microorganisms is inhibited. When the siderophore producing PGPR is present in rhizosphere it supplies iron to plants thereby stimulating plant growth.

4.4.4 Parasitism

Parasitism is a phenomenon where one organism consumes another organism, often in a subtle, non-debilitating relationship. These aspects are dealt with the example of fungi, nematodes and amoebae.

Table 1 Examples of Predation and Parasitism

Mode of Antagonism	Plant Pathogen	Antagonistic (hosts)	Post Infection Events
Mycoparasitism	*Rotrytisalii*	*Gliocladiumroseum*	Penetration of hyphae
	Cocchliobolus sativus	*Myrothecium verrucaria & Epicoccumpurpurascens*	Antibiosis and Penetration
	Rhizoctoniasolani & Fomesannosus	*Trichodermaviride*	Coiling, Cytoplasm Coagulation
	Sclerotiumrolfsii	*T. harzianum*	Coiling, Penetration and Lysis
Nematophagy	*Heterodera rostochiensis*	*Phialospora heteroderae*	Penetration of Cysts and Egg Killing
Mycophagy	*Cocchliobolus sativus*	Soil Amoebae	Perforation in Conidia
	Gaeumannomyces graminis var. tritici	Soil Amoebae	Penetration and Lysis of Hyphae

Table 1 Examples of Predation and Parasitism

4.4.4.1 Mycoparasitism

The mycoparasitism commonly occurs in nature by several methods which lead to predation viz., coiling, penetration, branching, sporulation, resting body production, barrier formation and lysis (Fig.1).

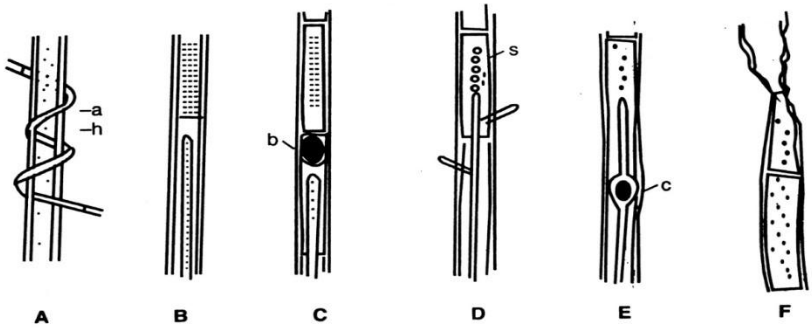


Fig. 1 Post hyphal interaction events during Mycoparasitism. A: Coiling (a-antagonist; h-host hypha); B: Penetration; C: Barrier Formation (c-by host); D: Branch Formation and Sporulation (s-by antagonist); E: Chlamydospore (c- formation); F: Lysis of host hypha (Dubey and Dwived, 1986).

4.4.5 Nematophagy

This is the phenomenon of eating upon nematodes by fungi. However, several nematode eating *i.e.* nematophagous fungi (NF) are known which develop different kinds of trap (T), arrest the pathogenic nematodes (N) and finally kill them. Morphological (Fig. 2) and biochemical aspects of trap formation are discussed by Cook (1977). Examples of nematode trapping fungi are *Arthrofyotrys, Dactylaria, Dactyleela*. *Phialospora heteroderae* penetrates the cysts and kills the eggs of *Heteroderaro stochiensis.*

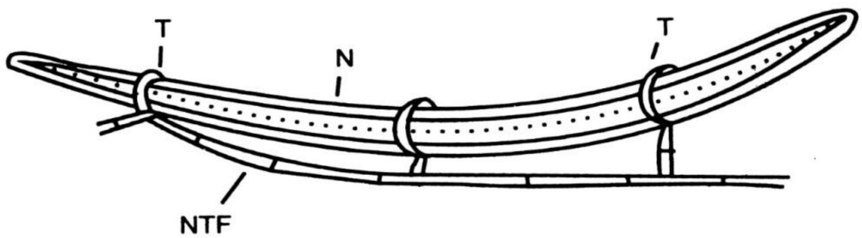


Fig: 2 Nematophagy N: a nematode; NTF: a nematode trapping fungus; T: a ring like trap formed by NTF.

Besides the fungi eating on nematodes, a spore forming bacterium, *Bacillus penetrans*kills the nematode and therefore, is used for the control of *Meloidogyne* sp. *B. penetrans* is resistant to nematicides. Being an obligate parasite, this bacterium cannot be grown in axenic culture. The bacterium shows host specificity and its spores survive for a long time. These spores adhere to the surface of infectious second-stage female larvae and eat on it. Adherance is followed by infection but it is not apparent until the adult stage.

4.5 CHECK YOUR PROGRESS QUESTIONS

1. What is Biopesticide?
2. Define Nematophagy
3. List the basic principle of Microbial Pesticides.
4. Siderophores
5. Amensalism

4.6 ANSWERS TO CHECK YOUR PROGRESS

1. Biopesticides are certain types of pesticides derived from natural materials such as animals, plants, bacteria and certain minerals.

2. The phenomenon of eating upon nematodes by fungi.

3. Antagonism, the action of any organism that suppresses or interferes with the normal growth and activity of a plant pathogen.

4. The extracellular metabolites which are secreted by bacteria.

5. It is an association between organisms of two different species in which one is inhibited or destroyed and the other remains unaffected.

4.7 SUMMARY

Biopesticides and its Types

Biopesticides are certain types of pesticides derived from natural materials such as animals, plants, bacteria and certain minerals. For example, canola oil and baking soda have pesticidal applications and are considered as biopesticides.

- ✓ Microbial Pesticides
- ✓ Plant Incorporated Protectants
- ✓ Biochemical Pesticides
- ✓ Botanical Pesticides

Microbial Pesticides

Microbial pesticides contain microorganisms (bacteria, virus, algae or fungi) as the active ingredient. It can control different types of pests, although each possesses separate active ingredients which are relatively specific for its target. For example there are fungi that control weeds and other fungi kill specific insects. They suppress pests by

producing such toxin specific action on the pest. Most common microbial pesticides are *Bacillus thuringiensis*.The greatest strength of microbial pesticides lies with their specificity as most are essentially non-toxic and non- pathogenic to the animals. Microbial pesticides include fungicides, herbicides, insecticides and growth regulators of microbial origin.Some of the important microbial pesticides are:

Bacillus thuringiensis - Discovered in Japan in early 20th century and became the first commercial product in France. It controls stem borer in rice, American bollworm in cotton. It releases toxins that damage the mid gut of pest.

Pseudomonas fluorescens **-** It has ability to grow quickly in the rhizosphere.

Trichoderma **-** It is a fungicide effective against soil borne disease such as in root nodes.

Agrobacterium radiobacter **-** It is used to treat roots while transplanting to check Crown gall. Crown gall is a disease which appears in the rose and other plants caused by soil borne pathogen, *Agrobacterium tumefaciens.*

Beautiful bassiana **-** It controls Colorado potato beetle.

Plant Incorporated Protectants (PIPs)

Plant Incorporated Protectants are the plants which are genetically engineered and they produce pesticides in their own tissue. Some specific genetic sequences when incorporated into a plant's genome can endow the plant with the ability to resist damage from certain pests.

A plant-incorporated protectant (PIP) is a pesticidal substance that is intended to be produced and used in a living plant or in the produce thereof and the genetic material necessary for production of such a pesticidal substance.

Biochemical Pesticides - Biochemical Pesticides are naturally occurring substances that control pests by non-toxic mechanisms. The substances may be plant extract, fatty acids or pheromones. It includes substances such as insect sex pheromones that interferes with mating as well as various scented plant extracts that attract insect pests to traps.

Botanical Pesticides - Naturally occurring plant materials like neem oil, rotenone, tobacco suspensions have the ability to control pest. It has insecticidal properties. They are toxic to the insect extracted or derived from the plants.The use of botanical insecticides to protect crops and stored products are as old as crop protection. Four major types of botanical insecticides are being used for insect control including pyrethrum, rotenone, neem and essential oils along with three others in limited use.

Basic Principle of Microbial Pesticide includes Antagonism, Parasitism, Amensalism, Siderophores, Parasitism and Nematophagy.

4.8 KEYWORDS

Integrated Pest Management, Plant Incorporated Protectants, Cytoplasmic Polyhedrosis Viruses, Nuclear Polyhedrosis Viruses, Biopesticides.

4.9 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Write a brief note on types of Microbial Pesticides
2. List out the biocontrol agents used to kill soil-borne pathogens
3. Account on Bacterial Pesticides
4. Comment on Siderophores
5. Define Amensalism

Long Answer Questions

1. Elaborate on the basic principles of Microbial Pesticides
2. Mention the Scope of Microbial Pesticides
3. In detail explain the types of Microbial Pesticides with suitable examples

4.10 FURTHER READINGS

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2. Handbook of Biofertilizers and Biopesticides. (2007), Edited by A.M. Deshmukh, R.M. Khobragade, P.P. Dixit.

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UNIT -V MICROBIAL HERBICIDES

Structure

5.1. INTRODUCTION

Microbial preparation of herbicide is defined as bioherbicides (Microbial herbicides) that can control the weed. The indigenous plant pathogens isolated from weeds are cultured to produce the large numbers of infective propagules which are applied at a rate that will cause high levels of infection leading to suppression of the target weed. A list of registered and commercially produced mycoherbicides is given in the Table 1. These plant pathogens based herbicide are generally evaluated for their virulence, performances under field condition and host range specificity.

5.2. OBJECTIVES

This unit includes the preparation of herbicides with their active sites having specific mode of actions. Bioherbicides can be prepared by solid and liquid formulation method. Hence we can understand the best way of formulation to control almost all weeds in agricultural field with all possible application.

Name of microbes	Name of Bioherbicide	Target weed species	Registration detail	Present Status
Acremonium diospyri	-	Persimmon trees in rangelands	USA, 1960	Unknown
Collectrotrichum gloeosporioides f. sp *cascutae*	Libao	*Cuscuta* sp.	Chaina, 1963	Probably still available
Phytopthora palmivora	DeVine™	*Morrenia odorata*	USA, 1981	Status unknown, may no longer be marketed
Collectrotrichum gloeosporioides f. sp. *aeschynomene*	Collego	*Aeschynomene virginica*	USA, 1983	No longer been available due to commercial backing
Puccina cnaliculata	Dr. BioSedge	*Cyperus esculantus*	USA, 1987	Registered, but product failed to uneconomic production system and resistance in some weed biotypes
Collectrotrichum gloeosporioides f. sp. *malvae*	BioMal™	*Malva pusilla*	Canada, 1992	Not commercially available but recently taken on by a new financial backer who is exploring market opportunities
Cylindrobasidium leave	Stumpout™	*Acacia* sp.	South Africa, 1997	Still available
Chondrostereum purpureum	Biochon™	*Prunus serotina*	Netherlands, 1997	Available until 2000, production stopped duc to low salc
Xanthromonas campestries pv. *poae*	Camperico™	*Poa annua*	Japan, 1997	Probably commercially available
Collectrotrichum acutatum	Hakatak	*Hakea gummosis* and *H. sericea*	South Africa, 1999	Not registered but will be produced on request
Puccinia thlaspeos	Woad Warrior	*Isastis tinctoria*	USA, 2002	Registered but commercially not available
Chondrostereum purpureum	Chontrol™ Ecoclear™	Alders and other hardwoods in rights of way and forests	Canada, 2004	Commercially available
Chondrostereum purpureum	Myco-Tech™	Deciduous tree species in rights of way and forests	Canada, 2004	Commercially available
Alterneria destruens	Smolder	*Dodder* species	USA, 2005	Just registered and company planning to do more field trials in 2007

Table: 1 Status of Microbial Commercial Bioherbicide since 1964.

5.3 MICROBIAL HERBICIDES

Bioherbicides are biocontrol agents applied to weeds in similar ways to conventional herbicides. The active ingredient in a bioherbicide is a living microorganism. Most commonly the organism is a fungus and hence the term Mycoherbicide is often used. Biological weed controls primarily the augmentation of indigenous fungal plant pathogens or the inundative approach has great potential to reduce chemical inputs and to provide viable, economic, effective weed control components within IPM programs.

Bioherbicides are applied in an attempt to overcome disease restraints by periodically dispersing an abundant supply of virulent inoculum onto a susceptible weed population. The application is timed to take advantage of favourable environmental conditions and/or the most susceptible stage of weed growth.

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5.3.1 Characteristics of Good Microbial Herbicides

A good Microbial Herbicide should possess the following characteristics.

i) They should be culturable in artificial media.
ii) They should be capable of abundant spore production.
iii) They should be stable in storage.
iv) They should be genetically stable.
v) They should be effective under field conditions.
vi) They should be tolerant to variations in temperature.

vii) They should be compatible with other chemicals/cultural practices.

5.3.2 Herbicide Types

Herbicides can be classified into several ways based on their effect, selectivity, persistence, application and action. They also can be divided further into types that are used for organic growing techniques and non-organic growing techniques.

(a) Effect

i) Pre-emergent Herbicides: Work as they interfere with seed germination. Pre-emergent need to be well-timed per the germination time of the weed that needs to be controlled. But when the timing is correct, they can provide the optimum solution to a weed problem. This type of herbicide is preventative rather than curative.
ii) Contact Herbicides: Works by killing all parts of the plant that they contact. In the majority of cases, these will not kill the whole plant and must be reapplied on a periodic basis. This will eventually drain the energy of the plan and kill it after the herbicide is applied several times.
iii) Systemic Herbicides: Kill the whole plant over a short period. Conducted throughout the plant in its vascular system. Many types of weeds are resistant to these forms of herbicides. Repeated applications could be needed.
iv) Drench Herbicides: Applied to soil instead of along with plant application. Most of these last a long time and are inappropriate for using where plants are grown.

(b) Selectivity

Selective herbicides are made to kill certain target plants and leave the plants that are desired unharmed. Herbicides that are used against lawn weeds are usually selective herbicides. Non-selective herbicides are made to kill any type of plant that they contact, but some plants are resistant to a variety of herbicides.

(c) Persistence

Many herbicides have different levels of persistence. This means that some are active for a longer period of time and others become inactive after they are applied in a short time.

(d) Application

Different herbicides may be applied in several ways. Some are liquid and are sprayed. Some are granules and some may be applied via an irrigation system or through fumigation. Some may even be painted directly onto the plants. The majority of herbicides are applied with several methods.

(e) Action

The major mechanisms of action for herbicides are:

- Dessicators: Work by the removal or water from the plant cells leading to the death of the plant.
- Acids and bases: Work like dessicators do by burning plant cells chemically. These are very acidic or alkaline substances.
- Nutritional controls: Work by shifting nutrient balance by providing too much of one nutrient or too much of another. This usually involves changing the pH of the soil but sometimes certain nutrients may be used to control certain types of plants.
- ACCcase inhibitors: Compounds that can kill varieties of grasses. ACCase inhibitors affect the cell membrane production in the grass plant meristems. ACCases of most grasses are sensitive to these types of herbicides.
- ALS inhibitors: This type of herbicide can slowly starve the affected plants of vital amino acids which can lead to the inhibition of DNA synthesis.
- EPSPS inhibitors: This type affects both grasses and dicots.
- Synthetic auxin: A form of organic herbicides that were discovered in the 40's after the study of plant growth.

5.3.3 Herbicide Mode and Site of Action

To be effective, herbicides must adequately contact plants, be absorbed by plants, move within the plants to the site of action without being deactivated and reach toxic levels at the site of action. The term "mode of action" refers to the sequence of events from absorption into plants to plant death or in other words, how an herbicide works to injure or kill the plant. The specific site the herbicide affects is referred to as the "site or mechanism of action." Understanding herbicide mode of action is helpful in knowing what groups of weeds are killed, specifying application techniques, diagnosing herbicide injury problems and preventing herbicide-resistant weeds.

- Auxin mimics (2,4-D, Clopyralid, Picloram and Triclopyr) which mimic the plant growth hormone auxin causing uncontrolled and disorganized growth in susceptible plant species.

- Mitosis inhibitors (Fosamine) which prevent re-budding in spring and new growth in summer (also known as dormancy enforcers).
- Photosynthesis inhibitors (Hexazinone) which block specific reactions in photosynthesis leading to cell breakdown.
- Amino acid synthesis inhibitors (glyphosate, imazapyr and imazapic), which prevent the synthesis of amino acids required for construction of proteins.
- Lipid biosynthesis inhibitors (fluazifop-p-butyl and sethoxydim) that s the synthesis of lipids required for growth and maintenance of cell membranes.

5.3.4 Formulations

The formulation of bioherbicide is the blending of the active ingredient, the biological propagules with a carrier or solvent and often other adjuvant to produce a form which can be effectively delivered to the target weed. Most of the formulations of the biological control agents are largely based upon techniques developed for formulation of agrochemical involving the use of organic solvents, surfactants and drying methods which can be detrimental to biological propagules.

Type of formulations		Substrate/medium of the bio-herbicide formulation
Liquid	Water	Distilled water (Connick *et al.*, 1990)
	Adjuvant	Bio-surfactants (e.g., surfactin by *Bacillus subtilis*), oxysorbic polyoxyethylene sorbitan monolauruate, oxysorbic polyoxyethylene sorbitan monoleate, polyglycerol etc. (Foy, 1989; Prasad, 1994)
	Inverted emulsions	Water suspended oil, soyabean lecithin, paraffinic oil, paraffinic soyabean oil etc. (Womack *et al.*, 1996; Yang and Jong, 1995).
	Oil suspension emulsion	Corn oil, coconut oil, wheat germ oil, cotton seed oil, fish oil, sunflower seed oil etc. (Auld, 1993; Egley and Boyette, 1995)
	Wettable powder	Aluminium- silicate powder. (Boyette *et al.*, 1996; Mortensen, 1998)
Solid	Alginate formulation	Sodium alginate, hydrous aluminium silicate etc. (Bashan, 1986)
	Other granular formulation	Corn starch, corn flour etc. (Boyette *et al.*, 1984; Zimmermann, 1993)

Table 2: Formulating substrate medium of potent bioherbicide

Majority of bioherbicide formulations are concentrated of maintaining agent viability in storage and reducing dew period requirements. Liquid and solid formulations of bioherbicides are the two different approaches for infection in above and below ground parts of the weed species.

(a) Liquid Formulation

Liquid formulation includes aqueous, oil or polymer based products, oil suspension emulsion, inverted emulsion etc. intended to be used as post emergence sprays to incite leaf and stem diseases on the weed host.Water is the simplest bioherbicide delivery system containingthe propagates of the agent formulated as spray able

suspension in water. Application of adjuvant for bioherbicide formulation assists or modifies the action of a principal active ingredient. This encompasses a wide range of compounds. A variety of microorganism produces some potent surfactants and can be used as biosurfactant in herbicide formulation. Application of adjutants in the formulation of herbicide sometimes cause up to 100% mortality of target weed within 48 hours. Yang and Jong (1995) prepared an inverted emulsion formulation of *Myrothecium verrucaria* by mixing and aqueous spore suspension with oil phase (1:1 v/v), where only oil emulsion carrier killed the 7 weed plants species. Auld (1993) developed an oil suspension emulsion formulation for control of *Xanthium spinosum*. Spores of *Colletotrichum orbicularae* were mixed with Kaolin (aluminium silicate powder) and dried. Dried powder (200 mg) was mixed with 20 mL of vegetable oils, 2 mL of an emulsifier and the water added to a volume of 200 mL. This formulation was tested in the field by Klein *et al*., (1995) and got up to 99% mortality in the first year. Two bioherbicides, Collego and BioMal were commercially available as a wettable powder. This formulation involves the drying of spores harvested from liquid fermentation together with a carrier such as Kaolin which can be stored before suspension in water.

(b) Solid Formulation

Fungal pathogen infect weeds at or below the soil are best studied to solid of granular formulations which may consist of grains, peat, charcoal, clay, vermiculite, alginate, bagasse, mineral soil or filter mud as carrier. These formulations of bioherbicides are better suited to pre-emergence applications, attacking weed seedlings as they emerge from the soil. Since granular formulations contain dried propagules, they may have a longer shelf life than liquid based formulations and is very important for a commercial bioherbicide.

During the last two decades, increasing interest has been observed on the synthetic beads of various materials for immobilization of herbicides, microorganism, cells and enzymes, antibodies, animal embryos and artificial seeds. Biodegradable slow release beads comprised of sodium alginate and skim milk were developed as carriers for the bacterial inoculation of plants. Walker (1981) developed a granular formulation of *Alterneria macrospora* for control of *Anoda cristata*. Mycelium of the pathogen was grown in liquid formulation, mixed with the horticultural vermiculite, exposed to diurnal light for 24 h to allow sporulation and air dried for 24-48 h. Field application of the granular inoculums incited almost 100% infection of *A. cristata* giving 75-95% control.

5.3.5 General Applications

Weeds are a constant problem for farmers. They not only compete with crops for water, nutrients, sunlight and space but also harbor insect and disease pests, clog irrigation and drainage system, undermine crop quality. Farmers fight weeds with tillage, hand weeding, synthetic herbicides, or typically a combination of all techniques. Similarly, many have argued that the heavy use of synthetic herbicides has led to groundwater contaminations, death of several wildlife species and has also been attributed to various human and animal illnesses. The use of bioherbicides is another way of controlling weeds without environmental hazards posed by synthetic herbicides. Bioherbicides are made up of microorganisms (e.g. bacteria, viruses, fungi).Bioherbicides deliver more of these pathogens to the fields. They are sent when the weeds are most susceptible to illness.

The genes of disease-causing pathogens are very specific. The microbe's genes give it particular techniques to overcome the unique defenses of one type of plant. They instruct the microbe to attack only the one plant species it can successfully infect. The invasion genes of the pathogen have to match the defense genes of the plant. Later the microbe knows it can successfully begin its attack on this one particular type of plant. The matching gene requirement means that a pathogen is harmless to all plants except the one weed identified by the microbe's genetic code. This selective response makes bioherbicides very useful because they kill only certain weed plants that interfere with crop productivity without damaging the crop itself. Bioherbicides can target one weed and leave the rest of the environment unharmed.

The benefit of using bioherbicides is that it can survive in the environment long enough for the next growing season where there will be more weeds to infect. It is cheaper compared to synthetic pesticides thus could essentially reduce farming expenses if managed properly. It is not harmful to the environment compared to conventional herbicides and will not affect non-target organisms.

With the advances of genetic engineering, new generation bioherbicides are being developed that are more effective against weeds. Microorganisms are designed to effectively overcome the weed's defenses. Weeds and not only to one type of weed as this can be too expensive to produce for commercial use. Microbes (plant pathogens) and microbial products (phytotoxins) have been shown to have potential as weed control agents. Growth in the interest in these alternative weed control methods has been brought about by a need for less persistent, more selective and more environmentally safe herbicides. Broad-spectrum and selective activities are concepts important to the development of a weed control agent. Generally,

pathogens are host-specific while phytotoxins have a wider range of activity.

Genetic engineering and microbial strain selection to increase pathogen virulence alter host range and enhance interactions with other chemical regulators or synergists may promote infectivity and weed control efficacy.

5.4 CHECK YOUR PROGRESS QUESTIONS

1. Main application of Microbial Herbicide
2. ACCcase Inhibitor
3. ALS inhibitors
4. Bioherbicide
5. Phytotoxin

5.5 ANSWERS TO CHECK YOUR PROGRESS

1. Weed Control.
2. Compounds that can kill varieties of grasses. They affect the cell membrane production in the grass plant meristems. ACCases of most grasses are sensitive to these types of herbicides.
3. This type of herbicide can slowly starve the affected plans of vital amino acids which can lead to inhibition of synthesis of DNA.
4. They consists of phytotoxins, pathogens and other microbes to be used as biological weed control.
5. They are substances that are poisonous or toxic to the growth of plants.

4.6 SUMMARY

✓ Bioherbicides are biocontrol agents applied to weeds in similar ways to conventional herbicides. The active ingredient in a bioherbicide is a living microorganism. Most commonly, the organism is a fungus and hence the term Mycoherbicide is often used.

✓ Herbicides can be classified in several ways: effect, selectivity, persistence, application and action. They also can be divided further types on the basis for usage of organic growing techniques and non-organic growing techniques.

✓ To be effective, herbicides must adequately contact plants, be absorbed by plants, move within the plants to the site of action without being deactivated, reach toxic levels at the site of action.

✓ Weeds are a constant problem for farmers. They not only compete with crops for water, nutrients, sunlight and space but also harbour insect and disease pests, clog irrigation and drainage system, undermine

crop quality. Farmers fight weeds with tillage, hand weeding, synthetic herbicides or typically with a combination of all techniques. Similarly, many have argued that the heavy use of synthetic herbicides has led to groundwater contaminations, death of several wildlife species and has also been attributed to various human and animal illnesses.

4.7 KEYWORDS

Microbial herbicides, EPSPS inhibitors, ACC ase

4.8 SELF ASSESSMENT QUESTIONS & EXCERCISE

1. Write short notes on Microbial Herbicide
2. Explain the types of Herbicides
3. Mention the mode of action of Bioherbicides
4. Discuss on the formulations of Bioherbicides
5. Applications of Bioherbicides

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UNIT -VI MICROBIAL INSECTICIDES

Structure

6.1 INTRODUCTION

Synthetic chemical insecticides which are popularly used nowadays impose serious hazards to human health and other organisms. Alternative method of insect management provides benefits to food production and human health. These alternatives are commonly referred to as integrated pest management, a strategy aimed at reduction on usage of chemical insecticides. One such alternative to chemical insecticide is the Microbial insecticide.

In this unit you will learn about the microbial insecticides that are commercially utilized nowadays prepared from bacteria, fungi and viruses along with its mode of action, benefits and limitations.

6.2 OBJECTIVES

To understand the mode of action of microbial insecticides and its applications.

6.3 MICROBIAL INSECTICIDES

Bacterial insecticides can be considered as a better alternative for the conventional chemical insecticides. With the use of bacterial insecticides, the food security and safety can be established. A wide range of bacterial pathogens are exploited for the production and formulation of bacterial insecticides. In general, the bacterial pathogens used for insect control are spore forming, rod shaped bacteria in the genus *Bacillus* and are isolated from soil samples. The organisms are classified into three types viz., obligate (*Bacillus lentimorbus* and *Bacillus papillae*), facultative (*Bacillus thuringiensis* and *Bacillus cerens)* and potential organisms (*Pseudomonas aeruginosa*). Around 100 potential exo and endo pathogens of bacteria against arthropods has been identified but only a few are commercially exploited. The bacterial insecticides provides a host specific insecticidal activity. Different bacteria are exploited against different host range.

Bacteria	Host
Bacillus thuringiensis var. *Kurstaki*	Larvae of moths and butterflies
Bacillus thuringiensis var. *Aizawai*	Wax moth Caterpillar
Bacillus lentimorbus and *Bacillus papillae*	Larvae of Japanese beetle
Bacillus sphaericus	Larvae of *Culex* and some *Aedes sp*.

Table 1: Bacterial species employed for insecticide production and their hosts

6.3.1 Bacterial Insecticides

(a) *Pseudomonas* sp.

Pseudomonas is Gram-negative, chemotropic and rod shaped motile bacteria. This group of bacteria are commonly known for their plant growth promotion and biological control of pathogens. Several species of Pseudomonas are exploited as biocontrol agents. Some of them include *P. aeruginosa, P. aureofaciens, P. chlororaphis, P. fluorescens and P. putida*. Among the Pseudomonas species, *P. fluorescens* are commonly utilized as insecticide. This bacterium utilizes a wide range of organic and inorganic compounds to live in adverse environmental conditions. It promotes biocontrol activity through direct antagonism of phytopathogens and induction of disease resistance in the host plant. It does not impose any adverse effects on environment and animal health.

(b) *Bacillus sp.*

Bacillus thuringiensis var *kurstaki*

It is soil borne bacteria used for controlling caterpillars, moths, hornworm and loopers. It exhibits higher insecticidal activity during 1st and 2nd instar stages of the caterpillar. It provides species specific insecticidal activity. It is a stomach toxin and it gets ingested by the host insect. It is applied through conventional spraying methods and is usually applied in the afternoon to maximize its effectiveness. It does not impose any adverse effects on humans and other beneficial insects.

Bacillus thuringiensis var *israelensis*

This bacterium offers higher insecticidal activity against mosquito and black fly. It exhibits insecticidal activity through a process called ingestion. Once the bacteria get ingested, it kills 95-100% of the larvae within 24 hours of time. It kills the insect pest before it reaches the adult stage. It is applied to irrigation areas and standing waters. No beneficial insects, humans and other vertebrates are harmed by this insecticide application.

Bacillus papillae

B. papillae is a Gram negative spore forming bacteria and is used to control beetle. It is an insect pathogen that can be effectively used as a microbial control agent. It is commercially available in powder form. The bacterial spores are powdered and applied over the soil. The spores get distributed naturally by rain and wind. It persists in the soil for over several years and kills the insect larvae.

Bacillus thuringiensis var *tenebrionis*

These bacteria are toxic to leaf eating beetle species and are considered as one of the most promising insecticide for pest control. It provides species specific toxicity and do not harm other beneficial insects. It is also safe to humans and do not impose adverse effects on the environment. It is mainly used to control elm leaf beetle. It can also be used to control pest on potatoes and tomatoes. It affects the pest insect at its larval stage and does not affect adult ones.

Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a soil borne bacteria used for natural insect control since 1950. Although bacteria like *B. papillae* and *B. sphaericus* are commercially exploited for insecticide production, their activity is limited when compared with *Bt*. There are many types of *Bt* that targets different insect groups such as mosquitoes, caterpillar

and moths. *Bt* is safe for humans and the *Bt* genes have been incorporated into several crops to make them insect resistant. This is achieved through genetic engineering technique. The first commercially produced insecticide based on *Bt* was sporine which is used against flour moths. The *Bt* is available in the market in powder form containing a mixture of dried spores and toxin crystals. The DNA of the *Bt* contains insect pathogen genes. It produces an insecticidal crystal protein called Cry toxins. The Cry toxin interacts with the midgut protein of the target insect and forms an oligomer structure. The osmotic shock created by these interactions kills the target insect. The *Bt* also produces a secondary metabolite called thuringiensin which also possess insecticidal activity. *Bt* toxin can be applied to potatoes, cotton and corn to protect the crops from pest insect.

Bt Cotton

BT cotton is an insect resistant transgenic crop formed by insertion of one or more genes into the soil borne bacterium called *Bacillus thuringiensis*. It is produced through genetic engineering techniques. Agrobacterium mediated gene transfer technique is commonly utilized for the production of transgenic cotton crop. The inserted gene is called as transgene. The genes inserted into the plant genome produce toxic crystals with insecticidal activity and are toxic to beetles, mosquitoes and caterpillar pests. The toxin ingested by the pest insect gets dissolved and becomes activated by the high pH environment of the gut. The activated toxin binds with cadherin like proteins found on the epithelial layer of the pest insect's midgut. This binding results in the formation of ion channels that makes the potassium ions to flow from the cells. The formation of ion channels and potassium efflux lyses the epithelial cells found on the lining of the host and leads to the death of the organism. The BT toxins are highly specific and are specific to selected number of arthropod species. The first BT cotton to be marketed was Bollgard cotton that contains a toxin called Cry 1Ac. The Cry 1Ac is toxic to tobacco budworm and pink bollworm. In India, few genes resistant against *Fusarium* and *Verticillium* wilts are isolated and are successfully transformed into cotton as a part of Integrated Pest Management. There are more than 200 different types of BT toxins and each toxic to different pest insects. There are several advantages over the use of BT cotton such as reduced pesticide use, improved crop management effectiveness, reduced production cost and high yield. It improves the soil quality and also reduces the use of fossil fuels. It reduces the environmental pollution and health hazards associated with the prolonged use of chemical insecticides.

6.3.1.1 Mode of Action

The insect consumes *Bt* toxin and spores which causes mortality. Inside the host insect, the *Bt* toxin dissolves in the gut that possess high pH and it becomes active. The toxin binds to specific receptors of the gut lining and attacks the gut cells of the insect to make it starve. The crystals break the gut wall which paves path for the entry of the spores and normal gut bacteria inside the host. The spores later germinate and the normal gut bacteria proliferate inside the body of the host insect and get killed.

6.3.1.2 Benefits

1. It is toxic to a narrow range of insects and does not harm beneficial insects.

2. *Bt* toxins consumed with GMO food crops are non-toxic to humans and other mammals.
3. Sustainable agriculture will be benefited by the prolonged application of Bt.

6.3.1.3 Limitations

1. It is more susceptible to degradation by sunlight.
2. New strains developed for leaf beetle control become ineffective after 24 hours of application.

6.3.2 Viral Insecticide

Microbial viral insecticides are pathogens that attacks and kills insects and other arthropods and hence are considered as a powerful tool of biological control. The viruses are capable of managing pest insect populations and are hence widely used for insecticide production. Sustainable agriculture will be benefited with the wide range use of viral insecticides. Among the insect viruses that are naturally found, the Baculoviruses have been commercially exploited for the production of viral insecticides. A number of features in the Baculoviruses make them an ideal one for insecticide production. The Baculoviruses possess high pathogenicity and virulence coupled with a long shelf life. These are safe for vertebrates and their host specificity is narrow that it often targets single insect species. Baculoviruses are isolated from Lepidoptera, Hymenoptera and Diptera species. These insecticides are easily applied using conventional spray equipments.

Virus	Insect Pest	Commodity
Codling moth granulosis virus	Codling moth	Apple and Pear
Autographa californica NPV	Alfalfa looper (*Autographa californica*)	Alfalfa crops
Lymantria dispar NPV	Gypsy moth (*Lymantria dispar)*	Lumber
Helicoverpa zea NPV	Tobacco budworm (*Helicoverpa zea)*	Cotton and vegetables
Anagrapha falcifera NPV	Celery looper (*Anagrapha falcifera)*	Vegetables

.**Table 2:** Virus employed for insecticide production and their hosts

Baculovirus as Insecticide

Baculovirus are extremely small in size and composed of double stranded DNA that encodes genes for virus establishment and reproduction. The Baculoviruses that are considered as potential candidate for insecticide production are in the genus of Nucleopolyhedrovirus. These viruses provide narrow and species specific insecticidal applications. The genetic material of the Baculovirus is easily destroyed when exposed to sunlight and also by the physical and chemical conditions inside the host gut. The genetic material is enclosed within the infective particle called virion. To tolerate the adverse circumstances, it is protected by a protein called polyhedron.

6.3.2.1 Mode of Action

The Baculovirus possess the insecticidal activity through a process called ingestion. It must be eaten by the host which produce an infection to kill the pest insect. Once ingested, the virus gets entered into the midgut of the insect larvae and the protein capsules of the virus get dissolved and release virions. The virions are the infective particles that start to destroy the midgut and other cells inside the host. The process continues for next few days and finally makes the larvae to die.

6.3.2.2 Benefits

1. Highly effective against a variety of insects and other arthropods.
2. Beneficial insects such as bees, mites and parasitoids are not harmed.
3. Highly specific and have a narrow host range.
4. It does not produce toxins or secondary compounds.
5. Humans, mammals and aquatic species are not affected.

NOTES

6.3.2.3 Limitations

1. High specificity of the Baculoviruses is considered as its limitation for its use in agricultural field as the farmers prefer usage of one product against a variety of pests.
2. Low persistence when exposed to UV light.

6.3.3 Fungal Insecticides
Entamopathogenic Fungi

Entamopathogenic fungi are fungal species that are pathogenic to insects. It regulates the insect pest population without affecting the non target insects and hence plays an important role in biological control. The entamopathogenic fungi infect insects of the orders: Homoptera (whiteflies and scale insects), Diptera (flies and mosquitoes), Coleoptera (beetles), Lepidoptera (particularly larvae), Orthoptera and Hymenoptera (bees). These fungi are commonly found in forest soils and are very effective in controlling *Ixodes scapularis* populations. Over 800 species of entamopathogenic fungi are indentified so far. Among them, the fungi belonging to the genera *Metarhizium* and *Beauveria* are commonly used as arthropod vector control tools. In the year 1835, Agostino Bassi formulated the use of white muscardine fungus on silkworm for disease management and the fungus was named after him as *Beauveria bassiana*. Later in 2010, Gilbert and Gil proposed the idea of using insect infecting fungi in insect pest control. The fungal based insecticide contains the parasitic fungi that grows inside an insect body and feeds on the internal tissues of the insect until it dies. These ready to use formulations are found available in many countries of Asia, Africa and Europe. The functioning of the fungal insecticide varies depending on the microorganism used for their production. Some repel the pest, while others affect the breeding process to control the growing population of the pest insect or even ends up with a specific disease to control the invader.

Fungus	Target Pest	Commodity
Beauveria bassiana	Whiteflies	Melon
Beauveria bassiana BB-01	*Lipaphis erysimi*	Canola (*Brassica napus*)
Verticillium lecanii	*Myzus persicae*	Chilli
Verticillium lecanii V17	Cabbage aphid	Cabbage
Cladossporium oxysporium	*Aphis craccivora* Koch	Cowpea

Table 3: Fungal species employed for insecticide production and their target pest

6.3.3.1 Mode of Action

The entamopathogenic fungi kill the insect by affecting the breeding ability of the insect, starvation and toxin production. The cuticle of the target insect hinders the penetration of the fungi into the body cavity thereby providing a physical defence to the target insect. For the penetration, the fungi produce some toxins and extracellular enzymes such as proteases and chitinases. The infective unit of most of the fungi is conidia. The conidia are adhesive to the cuticle of the pest insect. Under favourable condition, the conidium germinates and forms a structure called appressoria. The appressoria under suitable conditions attaches into the cuticle of the target insect and paves way for the easier penetration of the fungi into the body cavity of the insect. By enzymatic dissolution of chitin and protein, the fungal hyphae reach the internal organs. The insect virtually becomes filled with fungus and gets killed by the toxins produced by the fungi or by choking of the internal tissues by the fungal conidiophores.

6.3.3.2 Benefits of Entamopathogenic Insecticides

1. They are non-toxic to non-target organisms.
2. The residues of the insecticide do not impose any adverse effect on the environment.
3. These are specific to a single group or few species of insects.
4. They are self perpetuating.

6.3.3.3 Limitations

1. It is very costly to produce for commercial usage and have short shelf life.
2. Low rate of infection.
3. Special procedures are needed for their production.

6.4 CHECK YOUR PROGRESS QUESTIONS

1. Differentiate pesticide and insecticide
2. What is BT cotton?
3. Abbreviate IPM and define it

6.5 ANSWERS TO CHECK YOUR PROGRESS

1. Pesticides are chemicals that kill pathogenic fungi, virus, bacteria, insects and weeds. The insecticides are also a type of pesticide that specifically target and kills insects.

2. It is a transgenic crop produced with resistance to insects.
3. IPM stands for Integrated Pest Management. It is a combination of strategies aimed at reduction of chemical insecticide use.

6.6 SUMMARY

- India being an agricultural country faces many challenges in the production of food crops. The major hindrance for the production of such crops is the prolonged use of chemicals that affects the soil fertility and production rate.
- Microbial insecticides are considered as an ideal alternative to the chemical insecticides. The microbial insecticides are produced from microorganisms and hence are safe to humans and are eco friendly.
- The bacteria, virus and fungus are the common species of interest employed for insecticide production.
- The genetic engineering techniques are employed for the production of microbial insecticides.
- The three types of microbial insecticides target different species of pest insects and kill them effectively.
- Even though the microbial insecticides are found with enormous benefits, they do possess some limitations confined to the production cost and procedure, methods of application.

6.7 KEYWORDS

***Bacillus thuringiensis*, Cry toxin, Baculovirus**

6.8 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Define Microbial Insecticides
2. Write in brief about bacterial insecticides
3. Give a brief note on Entomopathogenic fungi
4. Mention the applications of Microbial Insecticides
5. Elucidate the structure of Baculovirus

Long Answer Questions

1. Explain in detail about *Bacillus thuringiensis*
2. Describe about BT cotton
3. Elaborate viral insecticides
4. Comment on bacterial species employed for insecticide production
5. In detail explain microbial insecticides

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UNIT -VII BIOLOGICAL CONTROL

Structure

7.1 INTRODUCTION

The terms of "biological control" and its abbreviated synonym "biocontrol" have been used in different fields of biology, most notably entomology and plant pathology. The biotechnological developments refer biological control as "the use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms and to favour desirable organisms such as crops, beneficial insects and microorganisms".

In entomology, the use of live predatory insects, entamopathogenic nematodes or microbial pathogens are used to suppress populations of different pest insects. In plant pathology, the term applies to the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control weed populations. In both fields, the organism that suppresses the pest or pathogen is referred to as the Biological Control Agent (BCA). More broadly, the term biological control also has been applied to the use of the natural products extracted or fermented from various sources.

7.2 OBJECTIVES

This unit provides descriptions of biological control (or biocontrol) agents of Plant pathogens and VAM fungi. It also includes the concept and practice of biological control, Pathogens-Antagonists and VAM fungi with its modification of culture practices.

7.3 Biological Control, Pathogen-Antagonists, VAM

7.3.1 Introduction of Antagonists

Antagonists are agents that can intensify microbial interaction resulting in control of disease and or disease-causing organism. A potential antagonist is isolated from a specialized niche (a habitat of organisms where they live and function with respect to other organisms and the environment), artificially, multiplied on nutrient media and introduced in the same habitat for microbial interactions and control of a particular disease. It is unlikely that an antagonist can be applied for a number of disease/pathogen in varied habitats. Antagonists can be introduced as a seed inoculums, vegetative part inoculation and soil inoculation.

7.3.2 Pathogen-Antagonists

Biological control of plant diseases including fungal pathogens has been considered a viable alternative method to chemical control. In plant pathology, the term biocontrol applies to the use of microbial antagonists to suppress diseases. Throughout their lifecycle, plants and pathogens interact with a wide variety of organisms. These interactions can significantly affect plant health in various ways. Different mode of actions of biocontrol-active microorganisms in controlling fungal plant diseases include hyperparasitism, predation, antibiosis, cross protection, competition for site and nutrient and induced resistance.

Type	Mechanism	Examples
Direct Antagonism	Hyperparasitism/Predation	Lytic/some nonlytic mycoviruses *Ampelomyces quisqualis* *Lysobacter enzymogenes* *Pasteuria penetrans* *Trichoderma virens*
Mixed-Path Antagonism	Antibiotics	2,4-diacetylphloroglucinol

		Phenazines Cyclic lipopeptides
	Lytic enzymes	Chitinases Glucanases Proteases
	Unregulated Waste Products	Ammonia CO_2, Hydrogen Cyanide
	Physical/Chemical Interference	Blockage of soil pores Germination signals consumption Molecular cross-talk confused
Indirect Antagonism	Competition	Exudates/leachates consumption Siderophore scavenging Physical Niche Occupation
	Induction of Host Resistance	Contact with fungal cell walls Detection of pathogen-associated, molecular patterns Phytohormone-mediated induction

Table 1: Types of Interspecies Antagonisms leading to Biological Control of Plant Pathogens

7.3.2.1 Types of Interactions Contributing to Biological Control

`Throughout their lifecycle, plants and pathogens interact with a wide variety of organisms which can significantly affect plant health in various ways. In order to understand the mechanisms of biological control, it is helpful to appreciate the different ways that organisms interact. The types of interactions include mutualism, protocol-operation, commensalism, neutralism, competition, amensalism, parasitism and predation. Examples of all of these types of interactions can be found in the natural world at both the macroscopic and microscopic level. The development of plant diseases involves both plants and microbes, the interactions that lead to biological control take place at multiple levels of scale. Significant biological control most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens.

7.3.2.2 Mechanisms of Biological Control

Pathogens are antagonized by the presence and activities of other organisms that they encounter. They assert that the different mechanisms of antagonism occur across a spectrum of directionality related to the amount of interspecies contact and specificity of the interactions (Table 1). Direct antagonism results from physical contact and/or a high degree of selectivity for the pathogen by the mechanism(s) expressed by the BCA(s). In, hyperparasitism by obligate parasites, a plant pathogen would be considered the most direct type of antagonism because the activities of no other organism would be required to exert a suppressive effect. In contrast, indirect antagonisms result from activities that do not involve sensing or targeting a pathogen by the BCA(s). Stimulation of plant host defence pathways by non-pathogenic BCAs is the most indirect form of antagonism.

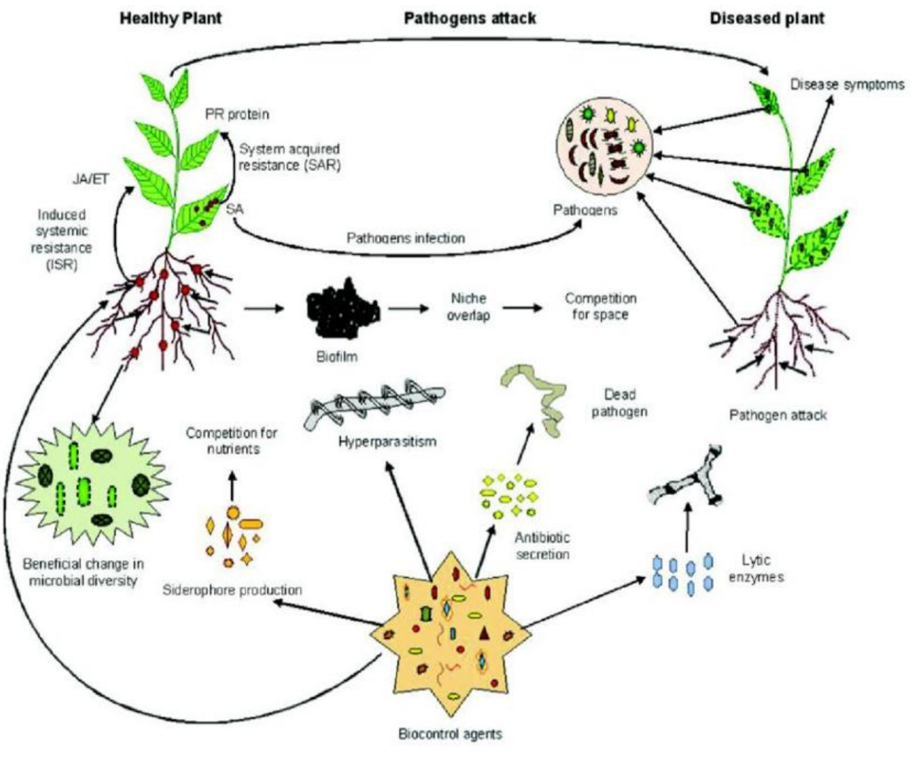


Figure 1: Mechanism of Biological Control in Plant

(a) Competition

Microorganism competes for space, minerals and organic nutrients to proliferate and survive in their natural habitats. This has been reported in both rhizospheres as well as phyllosphere. Competition has been suggested to play a role in the biocontrol of species of Fusarium and Pythium by some strains of Fluorescent Pseudomonas. Competition for substrates is the most important factor for heterotrophic soil fungi. Those fungi with the highest number of propagules or the greatest mass of mycelial growth have the greatest competitive advantage. Competitive Saprophytic Ability (CSA) is the summation of the physiological characteristics that make for success in competitive colonization of dead organic substrates.

(b) Antibiosis

Antibiosis is defined as antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds or other toxic substances. Antibiosis plays an important role in biological control. Antibiosis is a situation where the metabolites are secreted by underground parts of plants, soil microorganism, plant residues etc. It occurs when the pathogen is inhibited or killed by metabolic products of the antagonists. The products include the lyric agents, enzymes, volatile compounds and other toxic substances.

(c) Mycoparasitism/Hyperparasitism

Mycoparasitism or hyperparasitism occurs when the antagonist invades the pathogens by secreting enzymes such as chitinases, celluloses, glucanases and other lytic enzymes. Mycoparasitism is the phenomenon of one fungus being parasitic on another fungus. The parasitic fungus is called hyperparasite and the parasitized fungus as hyperparasite. In mycoparasitism, two mechanisms operate among involved species of fungi. This may be hyphal of interfungus interaction i.e., fungus-fungus interaction, several events take place which leads to predation viz., coiling, penetration, branching and sporulation, resting body production, barrier formation and lysis.

(d) Lytic Enzymes

Lysis is the complete or partial destruction of a cell by enzymes. Lysis may be distinguished into two types, endolysis and homolysis. Endolysis (autolysis) is the breakdown of the cytoplasm of a cell by the cell’s own enzymes following death which may be caused by nutrient starvation or by antibiosis or other toxins. Endolysis does not usually

involve the destruction of the cell wall. Exolysis (heterolysis) is the destruction of the cell by the enzymes of another organism. Typically homolysis is the destruction of the walls of an organism by chitinases, cellulases and this frequently results in the death of the attacked cell.

(e) Hydrogen Cyanide

Many rhizobacteria produce hydrogen cyanide and this has been shown to play direct as well as indirect role in biological control of plant diseases and increasing the yields. The Fluorescent Pseudomonas themselves produce HCN and are able to suppress the pathogens.

(f) Induced Systemic Resistance (ISR)

ISR is the ability of an agent (a fungus, bacteria, virus or chemical) to induce plant defence mechanisms that lead to systemic resistance to a number of pathogens. Inoculation of plants with weak pathogens or non-pathogens leads to induced systemic plant resistance against subsequent challenge by pathogens. The mechanisms remain largely unknown but typically, the induced resistance operates against a wide range of pathogens and can persist for 3-6 weeks. The biocontrol agents bring about induced systemic resistance (ISR) through fortifying the physical and mechanical strength of cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defence chemicals against challenge inoculation of pathogens. Defence reaction occurs due to accumulation of PR proteins (chitinase, B-1, 3 glucanase), chalcone synthase, phenylalanine ammonia lyase, peroxidase, phenolics, callose, lignin and phytoalexins.

(g) Plant Growth Promotion

Biocontrol agents also produce growth hormones like Auxins, Cytokinin, Gibberellins. These hormones suppress the deleterious pathogens and promote the growth of plants and simultaneously increase the yield. The studies on the mechanism of growth promotion indicated that PGPR promotes plant growth directly by production of plant growth regulators or indirectly by stimulating nutrient uptake by producing siderophores or antibiotics to protect the plant from soil-borne pathogens or deleterious rhizosphere organisms. Pseudomonas sp. may increase plant growth by producing Gibberellins-like substances for mineralizing phosphates.

Antibiotics	Source	Target Pathogen	Disease	Reference
2, 4-diacetyl-phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium* sp.	Damping off	Shanahan *et al.*, (1992)
Agrocin 84	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*	Crown gall	Kerr (1980)
Bacillomycin D	*Bacillus subtilis* AU195	*Aspergillus flavus*	Aflatoxin contamination	*Moyne et al., (2001)*
Bacillomycin Fengycin	*Bacillus amyloliquefaciens* FZB42	*Fusarium oxysporum*	Wilt	Koumoutsi *et al.*, (2004)
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*	Damping off	Islam *et al.*, (2005)
Gliotoxin	*Trichoderma virens*	*Rhizoctonia solani*	Root rots	Wilhite *et al.*, (2001)
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*	Fire blight	Sandra *et al.*, (2001)
Iturin A	*B. subtilis* QST713	*Botrytis cinerea* *R. solani*	Damping off	Paulitz and Belanger (2001), Kloepper *et al.*, (2004)
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidermatum*	Damping off	Leclere *et al.*, (2005)
Phenazines	*P. fluorescens* 2-79,30-84	*Gaeumannomyces graminis* var. *tritici*	Take-all	Thomashow *et al.*, (1990)
Pyoluteorin, pyrrolnitrin	*P. fluorescens* Pf-5	*Pythium ultimum* *R. solani*	Damping off	Howell and Stipanovic (1980)
Pyrrolnitrin, pseudane	*Burkholderia cepacia*	*R. solani* *Pyricularia oryzae*	Damping off and rice blast	Homma *et al.*, (1989)
Zwittermicin A	*Bacillus cereus* UW85	*P. medicaginis* *P. aphanidermatum*	Damping off	Smith *et al.*, (1993)

Table 2: Antibiotics Produced by BCAs

7.3.2.3 ADVANTAGES

(a) Biological control is less costly and cheaper than any other methods.
(b) They give protection to the crop throughout the crop period.
(c) They are highly effective against specific plant diseases.
(d) They do not cause toxicity to the plants.
(e) Application of biocontrol agents is safer to the environment and to the person who applies them.
(f) They multiply easily in the soil and leave no residual problem.
(g) Biocontrol agents can eliminate pathogens from the site of infection.
(h) Biocontrol agents not only control the disease but also enhance the root and plant growth by way of encouraging the beneficial soil microflora. It increases the crop yield also. It helps in the volatilization and sequestration of certain inorganic nutrients. For example *Bacillus subtilis* solubilizes the element, phosphorous and makes it available to the plant.
(i) Biocontrol agents are very easy to handle and apply to the target.
(j) They are easy to manufacture.

7.3.2.4 DISADVANTAGES

1. Biocontrol agents can only be used against specific diseases.
2. They are less effective than the fungicides.
3. Biocontrol agents have slow effect in the control of plant diseases.
4. At present, only few biocontrol agents are available for use and are available only in few places.
5. They are unavailable in larger quantities at present.
6. This method is only a preventive measure and not a curative measure.
7. Biocontrol agents should be multiplied and supplied without contamination and this requires skilled persons.
8. The shelf life of biocontrol agents is short. Antagonists, *Trichoderma viridae* is viable for four months and *Pseudomonas fluorescens* is viable for 3 months only.
9. The required amount of population of biocontrol agents should be checked at periodical interval and should be maintained at required level for effective use.
10. The efficiency of biocontrol agents is mainly decided by environmental conditions.
11. A biocontrol agent under certain circumstances may become a pathogen.

7.3.3 Mycorrhizae

Mycorrhizae form a network of filaments that associate with plant roots and draw nutrients from the soil that the root system would not be able to access otherwise. This fungus-plant alliance stimulates plant growth and accelerates root development. One kilometre of hyphae (fine filaments) may be associated with a plant growing in a one litre pot and it can access water and nutrients in the smallest pores in the soil. It also makes the plant less susceptible to soil-borne pathogens and to other environmental stresses such as drought and salinity. In return, the plant provides carbohydrates and other nutrients to the fungi. They utilize these carbohydrates for their growth and to synthesize and excrete molecules like glomalin (glycoprotein). The release of glomalin in the soil environment results in better soil structure and higher organic matter content.

7.3.3.1 Benefits of Mycorrhizae

- Produce more vigorous and healthy plants.
- Increase plant establishment and survival at seeding or transplanting.
- Increase yields and crop quality.
- Improve drought tolerance allowing the watering reduction.
- Enhance flowering and fruiting.
- Optimize fertilizers use, especially phosphorus.
- Increase tolerance to soil salinity.
- Reduce disease occurrence
- Contribute in maintaining soil quality and nutrient cycling.
- Contribute in controlling soil erosion.

7.3.4 VAM (Vesicular Arbuscular Mycorrhizae)

Vesicular Arbuscular Mycorrhizae (VAM) is the most abundant of a group of symbiotic fungi that infect plant roots. VA Mycorrhizae obtain carbon from their plant hosts and increase the nutrient and water uptake of their hosts. Some species of VA Mycorrhizae colonize only particular plant hosts while others are less specific. VA Mycorrhizae consists of both external and intraradical structures with the most distinctive structures occurring inside the root.

VAM is a fungus that penetrates the roots of a vascular plant in order to help them to capture nutrients from the soil. These fungi are scientifically well known for their ability to uptake and transport mineral nutrients from the soil directly into the host plant roots. Approximately 80% of known plant species including most economically important crops have a known symbiosis with them.

NOTES

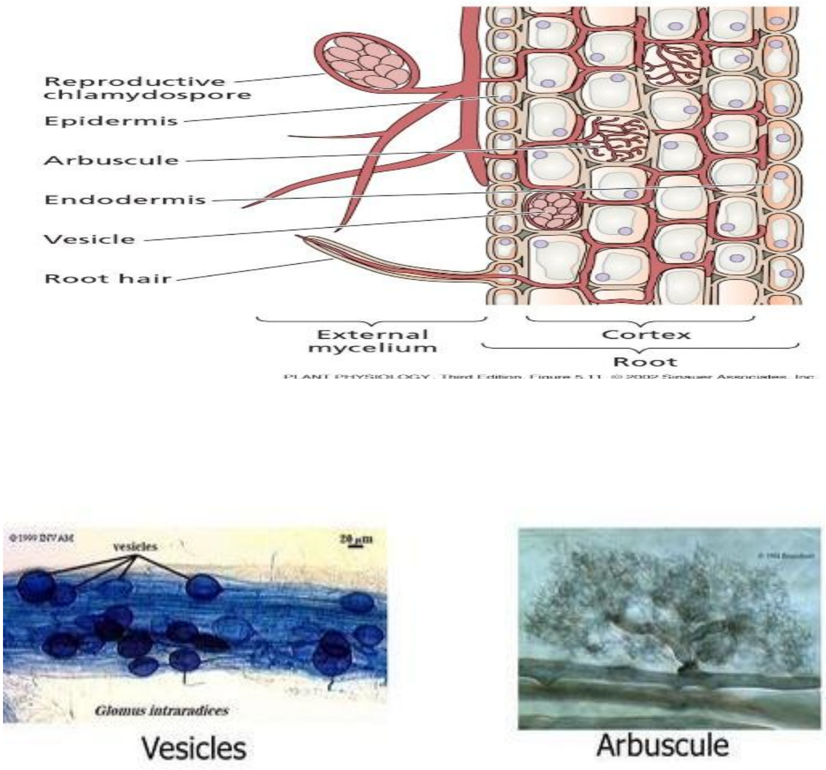


Fig. 3: VAM (Vesicular Arbuscule Mycorrhizae)

This mutual beneficial partnership between plants and soil fungi has existed as long as there have been plants growing in soil. Unfortunately, these beneficial Mycorrhizal fungi are destroyed in the development of human-made landscapes causing vegetation in these environments to struggle. When reintroduced to the soil the Mycorrhiza colonizes the root system forming a vast network of filaments. This fungal system retains moisture while producing powerful enzymes that naturally unlock mineral nutrients in the soil for natural root absorption.

NOTES

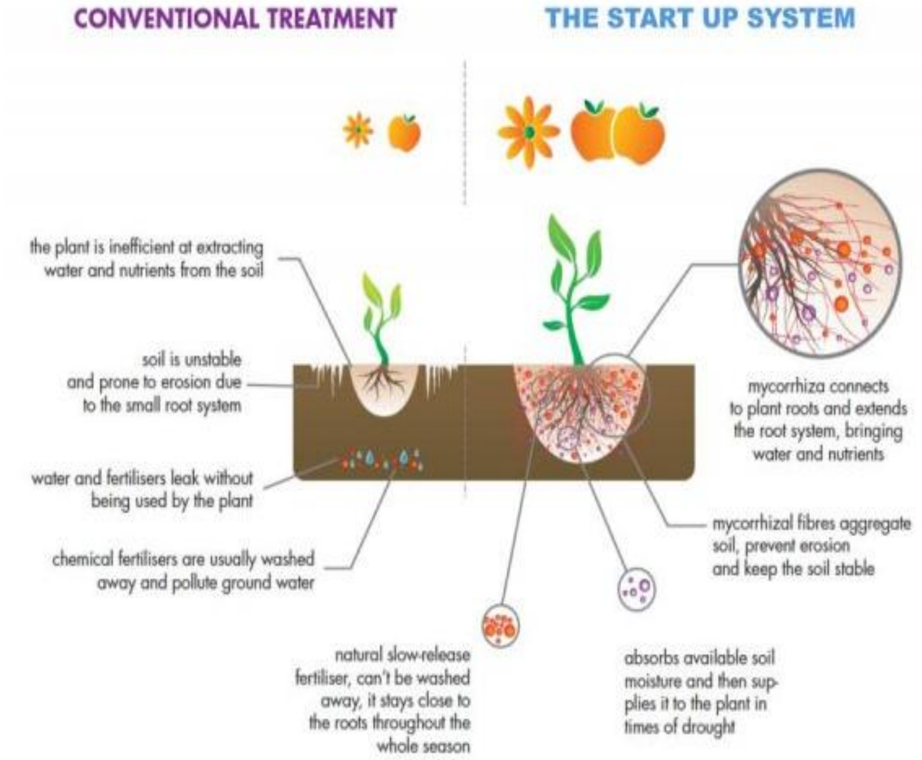


Fig 4: VAM Fungi Treatment in Plant Root

7.3.4.1 Microbial Diversity and Disease Suppression

Plants are surrounded by diverse types of mesofauna and microbial organisms, some of which can contribute to biological control of plant diseases. Microbes that contribute most to disease control are most likely those that could be classified as competitive saprophytes, facultative plant symbionts and facultative hyperparasites.

The epiphytes and endophytes may contribute to biological control. The ubiquity of Mycorrhizae deserves special consideration. Mycorrhizae are formed as the result of mutualist symbiosis between fungi and plants and occur on most of the plant species. Because they are formed early in the development of the plants, they represent nearly ubiquitous root colonists that assist plants with the uptake of nutrients especially phosphorus and micronutrients.

Arbuscular Mycorrhizae involve aseptate fungi and are named for characteristic structures like arbuscles and vesicles found in the root cortex. Arbuscles start to form by repeated dichotomous branching of fungal hyphae approximately two days after root penetration inside the root cortical cell.

Arbuscules are believed to be the site of communication between the host and the fungus. Vesicles are basically hyphal swellings in the root cortex that contain lipids and cytoplasm and act as a storage organ of VAM. These structures may be present intra and intercellular and can often develop thick walls in older roots. During colonization, VAM fungi can prevent root infections by reducing the access sites and stimulating host defence. VAM fungi have been found to reduce the incidence of root-knot nematode. Various mechanisms also allow VAM fungi to increase a plant's stress tolerance. This includes the intricate network of fungal hyphae around the roots which block pathogen infections.



Fig 5: Arbuscular Mycorrhizal Fungi as a Potential Tool in BioControl

7.3.4.2 Benefits from VAM Fungi

Soil Structure

VAM plays a vital role in maintaining soil quality through its impact on host plant physiology, ecological interactions and protection against causal microorganisms. Their filaments produce humic compounds and organic glues (extracellular polysaccharides) that bind soil into aggregates augmenting porosity. All these activities enhance aeration, water movements, root growth and distribution.

Plant Growth Hormones

Certain selective pores or seeds of VAM fungus possess growth-enhancing abilities. These inoculants are sprinkled onto roots during transplanting, worked into seed beds, watered in via existing irrigation methods and probed into the root zone of existing plants.

VAM contributes in increasing plant growth hormones like Cytokinins and Gibberllins.

Plant Roots

Due to morphological and physiological factors, VAM increases overall absorption capacity of the roots allowing a greater surface area for better utilization of low-availability of nutrients. It reduces reaction with soil colloids and leaching loss. Some of the trees like Pines cannot grow in new areas unless soil has Mycorrhizal inocula because of limited or coarse root hairs. Presence of VAM produces metabolites which change the ability of plants to induce roots from woody plant cuttings and increase root development during vegetative propagation.

Crop Yield

It is evident that VAM improves the yield amounts and quality due to translocation of immobile nutrients from soil to host plants including uptake of phosphorus. Availability of essential nutrients ensures better yield when compared to the conventional yield.

Nutrient Uptake

The symbiotic relationship allows the fungi to translocate immobile nutrients up to 12 cm away from the root surface. Along with the macronutrients like phosphorus, it enhances uptake of other micronutrients particularly zinc and copper. The mantle present in fungi also increases the rate of absorption of major and minor nutrients from the soil resulting in enhanced plant growth.

Disease and Pathogens

VAM fungi are recognized as high potential agents in plant protection and pest management. The mantle present in the fungi acts as a physical barrier against the invasion of root diseases. It secretes antibiotics that antagonize pathogens, aiding disease suppression.

7.4 MODIFICATION AND CULTURAL PRACTICED FOR REDUCING CROP DISEASES

Certain cultural practices are invaluable in reducing plant disease losses. A control program is enhanced whenever one can utilize as many methods of control as possible. New strains of an organism may develop that will attack resistant varieties or become tolerant to

certain pesticides when these practices are used alone. Combining practices reduces the risk of failure.

Rotation with unrelated crops is probably the most utilized cultural practice for disease control. This helps keep populations of pathogens from building up to damaging numbers. One should not expect rotation to eliminate disease development, but it certainly aids in reducing damage from most diseases.

Fertilizer usage may have some bearing on development of certain diseases. It differs with each crop and each disease but, in general, nitrogen out of balance with other nutrients enhances foliage disease development and predisposes some plants to other diseases. Potash, on the other hand helps reduce disease development when it is in balance with other elements.

Deep burial of crop residue helps control certain diseases by placing the organism contained in the residue at a depth where there is an oxygen deficiency. This reduces the population of the disease-causing organism and permits the crop to escape much of the damage.

Planting on a raised bed is helpful in preventing certain diseases such as Southern blight and certain of the wilt diseases. This practice is advisable when growing leguminous crops such as peanuts, soybeans, guar and when growing vegetable crops in tight, poorly drained soils.

Burning of crop residue has been discouraged because of destruction of valuable organic matter and creation of an air pollution problem. The fact remains, however, that it is a highly effective means of eradicating some disease-causing organisms associated with crop residue.

Time of seeding has an important bearing on disease prevention in many cases. Delayed planting of wheat will help escape the chances of wheat streak mosaic virus. Early spring planting of cotton may effectively help escape cotton root rot.

Removal of undesirable plants that might serve as a host reservoir for virus diseases that attack cultivated crops aid in preventing infection. Infected rhizomes of Johnson grass are the primary overwintering host for the maize dwarf mosaic virus that attacks grain sorghum, forage sorghum, and corn. Wild Solanaceae weeds such as Jimsonweed, Horsenettel and Silverleaf nightshade harbour viruses that attack potato and tomato.

Volunteer plants from a harvested crop are often means of carrying a disease organism from one crop season to the next. Rusts of cereal crops and spinach constitute an example of this type disease dissemination.

Roguing (removal) of diseased plants as they appear is often an effective method in helping reduce the spread of a destructive disease. Virus diseases of stone fruits and bacterial wilt of cucurbits are examples where roguing is worthy of consideration.

These cultural control practices have been found to be economically feasible in reducing disease losses. Growers should properly identify the diseases that limit production and use a variety of controls in combination.

7.5 CHECK YOUR PROGRESS QUESTIONS

1. Biological Control
2. Antagonist
3. Mycorrhizae
4. VAM
5. Mycoparasitism

7.6 ANSWERS TO CHECK YOUR PROGRESS

1. Biological Control is a method of controlling pests such as insects, mites, weeds and plant diseases using other organisms.
2. Antagonism refers to the action of any organism that suppresses or interfere the normal growth and activity of a plant pathogen, bacteria, fungi.
3. The term Mycorrhiza refers to the role of the fungus in the plant's rhizosphere, its root system.
4. VAM is Vesicular Arbuscular Mycorrhiza. It is formed by the symbiotic association between certain Phycomycetous Fungi and Angiosperm roots.
5. Mycoparasitism is the term used to indicate the interrelationships of a fungal parasite and a fungal host.

7.7 SUMMARY

- ✓ Cultural practices (e.g. good sanitation, soil preparation and water management) and host resistance can go a long way towards controlling many diseases.
- ✓ The greatest successes in biological control have been achieved in situations where the environmental conditions are most controlled.
- ✓ Biocontrol agents not only control the disease but also enhance the root and plant growth by way of encouraging beneficial soil microflora. It increases the crop yield also.
- ✓ Biocontrol agents are very easy to handle and apply to the target.

✓ They increase resistance in plants and with their presence reduce the effects of pathogens and pests on plant health.

✓ Some of them produce metabolites which change the ability of plants to induce roots from woody plant cuttings and increase root development during vegetative propagation.

7.8 KEYWORDS

- Biological Control, Pathogens, Antagonists, Vesicular Arbuscular Mycorrhizal fungi.

7.9 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Comment on Biological Control
2. Discuss the advantages and disadvantages of Biological control in plant field
3. Explain Mycorrhizal fungi
4. Mycoparasitism
5. How algae can be genetically engineered for enhancing photosynthetic efficiency?

Long Answer Questions

1. In detail explain the applications of Biological Control in plant field
2. Elaborate mechanism of Biological Control in plant with suitable diagram
3. List down the benefits of Mycorrhizae
4. Comment on Vesicular Arbuscular Mycorrhizae
5. Make a note on the modification of culture practices confined to VAM

7.10 FURTHER READINGS

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UNIT-VIII CYTOKINES

Structure

8.1 INTRODUCTION

Commercially Important Products

The genetically engineered cells have revolutionized the cell culture industry. Several specific promoters of human origin are utilized for high expression of foreign genes. For large scale production of certain biochemicals, the genetically engineered Baculovirus-infected animal cells are also in use in a bioreactor. To fulfil the process several perfusion systems have been developed that retain the cells in the bioreactor at the time of replacement of conditioned medium with fresh medium. This results in increase in cell density and in turn cell productivity. For commercial production of products, a large scale cell culture system and scaling up of process are required.

Some of the important products which are produced from animal cell cultures are: (i) enzymes (asperagenase, collagenase, urokinase, pepsin, hyaluronidase, rennin, trypsin, tyrosine hydroxylase), (ii) hormones (leutinizing hormone, follicle stimulating hormone, chorionic hormone and erythropoietin), (iii) vaccines (iv) monoclonal antibodies and (v) interferons.

8.2 OBJECTIVES

This Unit provides the reader with information regarding commercially important products likeHuman growth hormone. Finally the unit also covers important steps in Cytokines, Human growth hormones, Tissue plasminogen activator and Factor VII.

8.3 CYTOKINES, GROWTH HORMONES, TISSUE PLASMINOGEN ACTIVATOR, FACTOR VII

Cytokines

Cytokines are a large group of proteins that are secreted by specific cells of immune system. The term cytokine is made up of two parts: *cyto* (cell) and *kine* (movement). As signalling molecules, cytokines provide communication between cells and play a crucial role in modulating of the innate and adaptive immune response. Cytokines bind to specific receptors on the membrane of target cells, triggering signal-transduction pathways that ultimately alter gene expression in the target cells. The susceptibility of the target cell to a particular cytokine is determined by the presence of specific membrane receptors. Cytokines are produced by a broad range of cells including Interleukins, Lymphokines, Monokines, Interferons (IFN), colony stimulating factors (CSF), Chemokines and a variety of other proteins.Cytokines generally have a molecular mass of less than 30 kDa. The nomenclature of the cytokines was characterized by the secretion of the cells and the biological activity by four groups: the hematopoietin family, the interferon family, the chemokine family or the tumor necrosis factor family.This nomenclature is still used.

8.3.1 Properties of the Cytokines

There are several basic common properties of cytokines which are important in understanding their effect in the human body:

- Synthesis of cytokine is mainly induced by various stimuli which act on cells. They can also exist in preformed granules which are constitutively produced and secreted from cells.
- Cytokines achieve their effects by binding with high affinity to specific membrane receptors on cells. Therefore, cells show a relatively small number of specific cytokine receptors (100-1000 per cell). In other words, very low concentrations of cytokines can trigger biological effects in cells. Cellular response to the effects of cytokines is well regulated and it is reflected in the changes of gene expression in target cells resulting in the expression of new functions.
- Cytokines exert effects on different types of cells (the same cells express a variety of cytokine receptors), or one cytokine can exert many different biological effects. This cytokine action is

called pleiotropy. Also, several cytokines share the same functional effects and various cytokines can have the same or similar biological activity (various cytokines activate the same signalling pathways) which is called redundancy.

- Cytokines affect the synthesis and the activity of other cytokines, acting antagonistically, additively or synergistically.
- Cytokine activity can be autocrine (on the very cell that secretes it), paracrine (on surrounding cells) and endocrine (in distant cites from the production). Basic characteristics of cytokines suggest that their implementation achieves complex effects which are often accompanied by numerous side effects.

8.3.1.1Functional Group of Cytokines

Cytokine*	Secreted by**	Targets and Effects
Some Cytokines of Innate Immunity		
Interleukin 1 (IL-1)	Monocytes, Macrophages, Endothelial cells, Epithelial cells	Vasculature (inflammation); Hypothalamus (fever); Liver (induction of acute phase proteins)
Tumor Necrosis Factor(TNF)	Macrophages	Vasculature (inflammation); Liver (induction of acute phaseproteins); Loss of muscle, body fat (Cachexia); Induction of death in many cell types; Neutrophil Activation
Interleukin 12 (IL-12)	Macrophages, Dendritic Cells	NK cells; Influences Adaptive Immunity (Promotes TH_1 subset)
Interleukin 6 (IL-6)	Macrophages, Endothelial Cells	Liver (induces acute phase proteins); Influences Adaptive Immunity (Proliferation and Antibody secretion of B cell Lineage)
Interferon (IFN - α) (This is a family of molecules)	Macrophages	Induces an antiviral state in most nucleated cells; Increases MHC class I

		expression; activates NK Cells
Interferon (IFN-β)	Fibroblasts	Induces an antiviral state in most nucleated cells; increases MHC class I expression; activates NK cells.
SOME CYTOKINES OF ADAPTIVE IMMUNITY		
Interleukin 2 (IL-2)	T cells	T-cell proliferation; can promote AICD. NK cell activation and proliferation; B-cell proliferation
Interleukin 4 (IL-4)	TH2 cells; Mast cells	Promotes TH_2 differentiation; isotype switch to IgE
Interleukin 5 (IL-5)	TH_2 cells	Eosinophil Activation and Generation
Interleukin 25 (IL-25)	Unknown	Induces secretion of TH_2 cytokine profile
Transforming Growth Factor(TGF)	T cells, Macrophages, other cell types	Inhibits T-cell proliferation and effector functions; inhibits B-cell proliferation; promotes isotype switch to IgE; inhibits macrophages
Interferon (IFN)	TH1 cells; CD8+ cells; NK cells	Activates Macrophages; increases expression MHC class I and class II molecules; increases antigen presentation

. Many cytokines play roles in more than one functional category.

*Only the major cell types providing cytokines for the indicated activity are listed; other cell types may also have the capacity to synthesize the given cytokine.

**Also note that the activated cells generally secrete greater amounts of cytokine than the inactivated cells.

8.3.1.2 Cytokine Receptors

To exert their biological effects, cytokines must first bind to specific receptors expressed on the membrane of responsive target cells. Because these receptors are expressed by many types of cells, the cytokines can affect a diverse array of cells. Biochemical characterization of cytokine receptors initially progressed at a very slow pace because their levels on the membrane of responsive cells are quite low. Cytokine receptors have led to rapid advances in the identification and characterization of these receptors.

Receptors for the various cytokines are quite diverse structurally, but almost all belong to one of five families of receptor proteins.

- Immunoglobulin superfamily receptors
- Class I cytokine receptor family (also known as the hematopoietin receptor family)
- Class II cytokine receptor family (also known as the interferon receptor family)
- TNF receptor family
- Chemokine receptor family

8.3.1.3 Production and Medical Use of Cytokines

(a) Cytokine Immunotherapy

Immunotherapy is a medical term defined as the "treatment of disease by inducing, enhancing or suppressing an immune response". The active agents of immunotherapy are collectively called immunomodulators. They are a diverse array of recombinant, synthetic and natural preparations often cytokines such as granulocyte colony-stimulating factor (G-CSF), interferons, imiquimod and cellular membrane fractions from bacteria are already licensed for use in patients. Others including IL-2, IL-7, IL-12, various chemokines, synthetic cytosine phosphate-guanosine (CpG), oligodeoxynucleotides and glucans are currently being investigated extensively in clinical and preclinical studies.

1. INF α: Recombinant DNA technology in *E. coli* (Pegylation). It is used in the treatment of hepatitis B and C, chronic myeloid leukemia, malignant melanoma, non-Hodgkin’s lymphoma, Kaposi’s sarcoma and other diseases.

2. INF β: Recombinant DNA technology in *E. coli* and CHO cell line is mostly used in their production and is used in the treatment of multiple sclerosis.

3. INF γ: Recombinant DNA technology in bacteria,*E. coli*. It is used in the treatment of chronic granulomatous disease.

4. TNF (TumorNecrosis Factor): Recombinant DNA technology in *E. coli*. It effects in inflammation, infection and response to tumors.

5. IL-: Production of neutrophils and platelets in the bone marrow. The proinflammatory cytokine which stimulates the synthesis of substances involved in the induction of inflammation.

(b) Growth Hormone (Somatotropin)

It is a protein hormone of about 190 amino acids that is synthesized and secreted by cells called somatotrophs in the anterior pituitary. It is a major participant in the control of several complex physiologic processes including growth and metabolism. Growth hormone is also of considerable interest as a drug used in both humans and animals.

(i) Physiologic Effects of Growth Hormone

A critical concept in understanding growth hormone activity is that it has two distinct types of effects:

- Direct Effects are the result of growth hormone binding its receptor on target cells. Fat cells (adipocytes), for example have growth hormone receptors and growth hormone stimulates them to break down triglyceride and suppresses their ability to take up and accumulate circulating lipids.
- Indirect Effects are mediated primarily by an insulin-like growth factor-I (IGF-I), a hormone that is secreted from the liver and other tissues in response to growth hormone. A majority of the growth promoting effects of growth hormone is actually due to IGF-I acting on its target cells. Keeping this distinction in mind, we can discuss two major roles of growth hormone and its minion IGF-I in physiology.

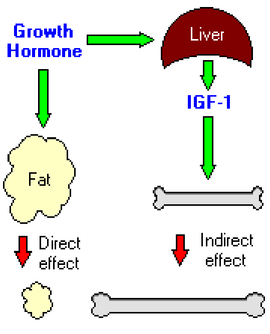


Fig. 1: Physiologic Effects of Growth Hormone

(c) Effects on Growth

Growth is a very complex process and requires the coordinated action of several hormones. The major role of growth hormone in stimulating body growth is to stimulate the liver and other tissues to secrete IGF-I. IGF-I stimulates proliferation of chondrocytes (cartilage cells) resulting in bone growth. Growth hormone does seem to have a direct effect on bone growth in stimulating differentiation of chondrocytes.

IGF-I also appears to be the key player in muscle growth. It stimulates both the differentiation and proliferation of myoblasts. It also stimulates amino acid uptake and protein synthesis in muscle and other tissues.

(d) Metabolic Effects

Growth hormone has important effects on protein, lipid and carbohydrate metabolism. In some cases, a direct effect of growth hormone has been clearly demonstrated. IGF-I is thought to be the critical mediator and in some cases it appears that both direct and indirect effects are at play.

(i) Protein Metabolism: In general, growth hormone stimulates protein anabolism in many tissues. This effect reflects increased amino acid uptake, increased protein synthesis and decreased oxidation of proteins.

(ii) Fat Metabolism: Growth hormone enhances the utilization of fat by stimulating triglyceride breakdown and oxidation in adipocytes.

(iii) Carbohydrate Metabolism: Growth hormone is one of a battery of hormones that serves to maintain blood glucose within a normal range. Growth hormone is often said to have anti-insulin activity because it suppresses the abilities of insulin to stimulate uptake of glucose in peripheral tissues and enhance glucose synthesis in the liver. Somewhat paradoxically, administration of growth hormone stimulates insulin secretion leading to hyperinsulinemia.

(e) Control of Growth Hormone Secretion

Production of growth hormone is modulated by many factors including stress, exercise, nutrition, sleep and growth hormone itself. However, its primary controllers are two hypothalamic hormones and one hormone from the stomach:

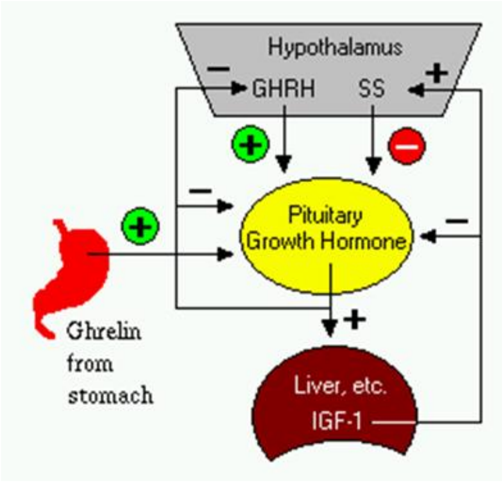


Fig. 2: Growth Hormone Secretion

- Growth hormone-releasing hormone (GHRH) is a hypothalamic peptide that stimulates both the synthesis and secretion of growth hormone.
- Somatostatin (SS) is a peptide produced by several tissues in the body including the hypothalamus. Somatostatin inhibits growth hormone release in response to GHRH and to other stimulatory factors such as low blood glucose concentration.
- Ghrelin is a peptide hormone secreted from the stomach. Ghrelin binds to receptors on somatotrophs and potently stimulates secretion of growth hormone.
- Growth hormone secretion is aalso part of a negative feedback loop involving IGF-I. High blood levels of IGF-I led to decreased secretion of growth hormone not only by directly suppressing the somatotroph, but by stimulating release of somatostatin from the hypothalamus.
- Growth hormone also feeds back to inhibit GHRH secretion and probably has a direct (autocrine) inhibitory effect on secretion from the somatotroph.
- Integration of all the factors that affect growth hormone synthesis and secretion led to a pulsatile pattern of release. Basal concentrations of growth hormone in blood are very low. In children and young adults, the most intense period of growth hormone release is shortly after the onset of deep sleep.

8.3.1.4 States of Diseases

- States of both growth hormone deficiency and excess provide very visible testaments to the role of this hormone in normal physiology. Such disorders can reflect lesions in either the hypothalamus, the pituitary or in target cells. A deficiency state

can result not only from a deficiency in production of the hormone, but in the target cell's response to the hormone.

- Clinically, deficiency in growth hormone or defects in its binding to receptor are seen as growth retardation or dwarfism. The manifestation of growth hormone deficiency depends upon the age of onset of the disorder and can result from either heritable or acquired disease.
- The effect of excessive secretion of growth hormone is also very dependent on the age of onset and is seen as two distinctive disorders:
- Gigantism is the result of excessive growth hormone secretion that begins in young children or adolescents. It is a very rare disorder, usually resulting from a tumor of somatotropes. One of the most famous giants was a man named Robert Wadlow. He weighed 8.5 pounds at birth, but by 5 years of age he was 105 pounds and 5 feet 4 inches tall. Robert reached an adult weight of 490 pounds and 8 feet 11 inches in height. He died at age 22.
- Acromegaly results from excessive secretion of growth hormone in adults usually the result of benign pituitary tumors. The onset of this disorder is typically insideous occurring over several years. Clinical signs of acromegaly include overgrowth of extremities, soft-tissue swelling, abnormalities in jaw structure and cardiac disease. The excessive growth hormone and IGF-I also led to a number of metabolic derangements including hyperglycemia.

8.3.1.5 Growth Hormone

- ➢ In years past, growth hormone purified from human cadaver pituitaries was used to treat children with severe growth retardation. More recently, the virtually unlimited supply of growth hormone produced using recombinant DNA technology has led to several other applications to human and animal populations.
- ➢ Human growth hormone is commonly used to treat children of pathologically short stature. There is concern that this practice will be extended to treatment of essentially normal children the so called "enhancement therapy" or growth hormone on demand. Similarly, growth hormone has been used by some to enhance atheletic performance. Although growth hormone therapy is generally safe, it is not as safe as normal therapy and does entail unpredictable health risks. Parents that request growth hormone therapy for children of essentially normal stature are clearly misguided.

➢ The role of growth hormone in normal aging remains poorly understood, but some of the cosmetic symptoms of aging appear to be amenable to growth hormone therapy. This is an active area of research and additional information and recommendations about risks and benefits will undoubtedly surface in the near future.

- Growth hormone is currently approved and marketed for enhancing milk production in dairy cattle. There is no doubt that administration of bovine somatotropin to lactating cow's results in increased milk yield and depending on the way the cows are managed can be an economically-viable therapy. However, this treatment engenders abundant controversy, even among dairy farmers. One thing that appears clear is that drinking milk from cattle treated with bovine growth hormone which does not pose a risk to human health.
- Another application of growth hormone in animal agriculture is treatment of growing pigs with porcine growth hormone. Such treatment has been demonstrated to significantly stimulate muscle growth and reduce deposition of fat.

8.3.1.6 Tissue Plasminogen Activator

Tissue Plasminogen Activator (tPA) is classified as a serine protease (enzymes that cleave peptide bonds in proteins) and is thus one of the essential components of the dissolution of blood clots. Its primary function includes catalyzing the conversion of plasminogen to plasmin, the primary enzyme involved in dissolving blood clots.

Recombinant Biotechnology has allowed tPA to be manufactured in labs and these synthetic products are called recombinant tissue Plasminogen Activator (rtPA). Examples of these drugs include alteplase, reteplase and tenecteplase. These drugs have undergone various modifications to amplify their pharmacokinetic and pharmacodynamic properties especially to prolong their short half-life in the circulation and further increase their fibrin specificity to prevent an unwanted fibrinolytic state.

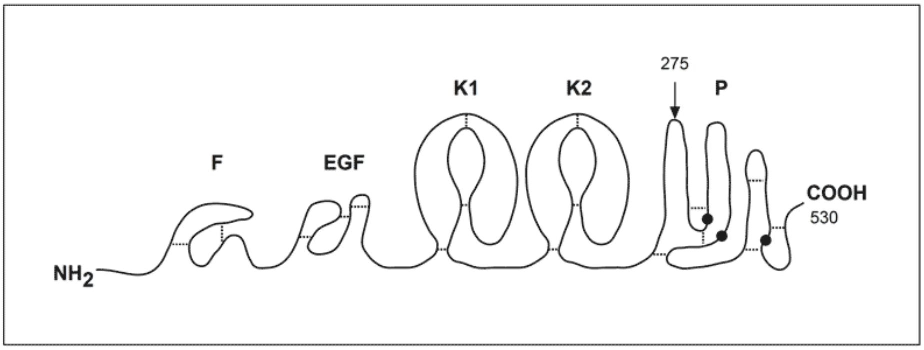
Alteplase is the normal, human plasminogen activator and is FDA approved for the management of patients with ischemic stroke, myocardial infarction with ST-elevation (STEMI), acute massive pulmonary embolism and those with central venous access devices (CVAD).

Reteplase is a modified form of human tPA with similar effects but a faster onset and longer duration of action. It is currently FDA approved for the management of acute myocardial infarction. It is preferred over alteplase due to its longer half-life and is allowed which allows it to be given as a bolus injection rather than through an infusion like Alteplase. Tenecteplase is another modified version of tPA with a longer half-life. It is indicated for the management of acute myocardial infarction (AMI).

Structure and Function of t-PA

Human t-PA is a 68 kDa serine protease composed of 530 or 527 amino acids containing between seven and 13% carbohydrate. The molecule is made up of five distinct domain structures with autonomous functions.

The finger domain and the two kringle domains are involved in the binding of t-PA to fibrin whereas the epidermal growth factor domain is implicated in the rapid hepatic clearance of the molecule. The serine protease domain contains the active site region made up of serine, histidine and aspartic acid which are situated relatively far apart from each other in the primary structure but are in close proximity in the folded protein. This region cleaves the Arg561-Val562 bond in plasminogen and thus activates it to plasmin as thin lines domain structure of the human t-PA molecule. Abbreviations: F; finger domain K1-2; kringle domains, EGF; epidermal growth factor domain, P; serine protease domain. The catalytic triad made up of His322, Asp371 and Ser478 is illustrated by black dots. The cleavage site for converting the molecule into the two-chain form is indicated by an arrow. Disulfide bridges are marked.



(a) Synthesis and Secretion

t-PA is synthesized mainly in the vascular endothelial cells and is secreted into the plasma continuously and also through the acute release of t-PA. The latter situation occurs upon stimulation of certain endothelial cell receptors. Different regions of the vascular system secrete different amounts of t-PA. Upper extremities secrete about four times more t-PA than that of the lower extremities.There are two forms of t-PA, single-chain t-PA (sct-PA) and two-chain t-PA (tct-PA). The single chain molecule is the native form of t-PA secreted from endothelial cells whereas the two-chain form is the result of the proteolytic activity of plasmin. Both forms are catalytically active and have similar enzymatic properties in the presence of fibrin.

Mechanism of Action

tPA is a thrombolytic (it breaks up blood clots) formed by aggregation of activated platelets into fibrin meshes by activating plasminogen. More specifically, it cleaves the zymogen plasminogen at its Arg561-Val562 peptide bond to form the serine protease, plasmin. Plasmin, an endogenous fibrinolytic enzyme breaks the cross-links between fibrin molecules which are the structural support of the blood clot and its activity is extremely short-lived. This is because alpha 2-antiplasmin, an abundant inhibitor of plasmin quickly inactivates it and restricts the action of plasmin to the vicinity of the clot.

The following sequence summarizes the action of tPA:

- tPA attaches to the fibrin on the clot surface.
- It activates the fibrin-bound plasminogen.
- Plasmin is subsequently cleaved from the plasminogen affiliated with the fibrin.
- The molecules of fibrin are broken up by the plasmin, and the clot dissolves.
-

Plasminogen activator inhibitor 1 (PAI 1) eventually terminates the catalytic activity of tPA by binding to it and this inactive complex (PAI 1-bound tPA) is removed from the circulation by the liver, more specifically via the scavenger receptor, LDL receptor-related protein 1 (LRRP1). In the nervous system, a neuronal-specific inhibitor of tPA, neuroserpin acts similarly to PAI 1 and the inactive tPA-neuroserpin complexes are internalized by the LRRP1 for removal from circulation.

(1) Toxicity

The drug used to reverse tPA toxicity is aminocaproic acid, an FDA-approved drug for the management of acute bleeding caused by increased fibrinolytic activity. It acts as an effective inhibitor for proteolytic enzymes like plasmin which is the primary enzyme responsible for fibrinolysis.

(2) Drug Interactions

Monitor closely with any drug that causes anticoagulation as there is an increased risk of bleeding.

Defibrotide: Through pharmacodynamic synergism, defibrotide increases the effects of tPA drugs and is thus contraindicated.

Prothrombin complex concentrate, human: This can cause pharmacodynamic antagonism of the tPA drugs.

Apixaban: Apixaban and tPA drugs increase anticoagulation and can lead to an increased bleeding risk.

Nitroglycerin: This could decrease the serum concentration of tPA drugs.

Salicylates: These could enhance the toxic effects of thrombolytic drugs. Monitor therapy as there is an increased risk of bleeding.

8.3.1.7 Factor VII (Labile Factor or Proconvertin)

Factor VII (FVII) is a 50-kiloDalton, vitamin K dependent, serine protease that is produced in the liver and circulates in the blood at a concentration of 0.5 μg/ml. The half-life of FVII is 3-4 hours. It's one of about 20 clotting factors involved in the complex process of blood clotting. FVII deficiency is quite rare with a prevalence of approximately 1:500,000. Homozygotes and compound heterozygotes often have FVII levels 0.01-0.03 U/ml and may have a severe bleeding phenotype, while heterozygotes with levels approaching 0.5 U/ml are typically asymptomatic.

(a) Role of Factor VII in Normal Blood Clotting

The normal blood clotting process occurs in four stages:

1. Vasoconstriction

When a blood vessel is cut, the damaged blood vessel immediately constricts to slow blood loss. Later the injured blood vessel releases a protein called tissue factor into the bloodstream. The release of tissue factor acts like an SOS call, signalling blood platelets and other clotting factors to report to the scene of the injury.

2. Formation of a Platelet Plug

Platelets in the bloodstream are the first to arrive at the injury site. They attach themselves to the damaged tissue and to each other forming a temporary, soft plug in the wound. This process is known as primary hemostasis.

3. Formation of a Fibrin Plug

Once the temporary plug is in place, the blood clotting factors go through a complex chain reaction to release fibrin, a tough, stringy protein. Fibrin wraps itself in and around the soft clot until it becomes a

tough, insoluble fibrin clot. This new clot seals the broken blood vessel and creates a protective covering for new tissue growth.

4. Wound Healing and Destruction of the Fibrin Plug

After a few days, the fibrin clot starts to shrink, pulling the edges of the wound together to help new tissue grow over the wound. As the tissue is rebuilt, the fibrin clot dissolves and is absorbed.If factor VII does not function properly or present in too little amount, the stronger fibrin clot cannot form properly.

(b) Factor VII Deficiency

It is an inherited bleeding disorder that is caused by a problem with roper working nature of the factor the clotting reaction is blocked prematurely and the blood clot does not form.
Factor VII deficiency is an autosomal recessive disorder which means that both parents must carry the defective gene in order to pass it on to their child. It also means that the disorder affects both males and females.
Factor VII deficiency may be inherited with other factor deficiencies.

Treatment
There are several treatments available for factor VII deficiency.

1. Recombinant VIIa Concentrate (rFVIIa)
2. Factor VII Concentrate
3. Prothrombin Complex Concentrate (PCC) containing factor VII
4. Fresh Frozen Plasma (FFP)

Excessive menstrual bleeding in women with factor VII deficiency may be controlled with hormonal contraceptives (birth control pills), intra-uterine drugs (IUDs) or antifibrinolytic drugs.

8.4 CHECK YOUR PROGRESS QUESTIONS

1. Cytokines
2. Somatotropin
3. Tissue Plasminogen Activator
4. Factor VII
5. Blood Clotting

8.5 ANSWERS TO CHECK YOUR PROGRESS

1. .Cytokines are a large group of proteins, peptides or glycoproteins that are secreted by specific cells of immune system.
2. Somatotropin also known as human growth hormone (hGH or HGH) in its human form is a peptide hormone that stimulates growth, cell reproduction and cell regeneration in humans and other animals.

3. Tissue Plasminogen Activator (tPA, tissue-type plasminogen activator) is a serine protease found on endothelial cells (cells that line the blood vessels) involved in the breakdown of blood clots (fibrinolysis). tPA enzyme catalyzes the conversion of plasminogen to plasmin.
4. Factor VII (FVII) is a 50-kiloDalton, vitamin K dependent, serine protease that is produced in the liver and circulates in the blood.
5. A blood clot is a clump of blood that has changed from a liquid to a gel-like or semisolid state. Clotting is a necessary process that can prevent you from losing too much blood in when injured or cut.

8.6 SUMMARY

- ✓ Human growth hormone is commonly used to treat children of pathologically short stature.
- ✓ Growth hormone is currently approved and marketed for enhancing milk production in dairy cattle. There is no doubt that administration of bovine somatotropin to lactating cows results in increased milk yield and depending on the way the cows are managed can be an economically-viable therapy.
- ✓ Cytokine therapies requires detailed animal toxicity studies with an increasing need for more specific tests for detecting changes in immune status such as immune deficiency, auto-immunity or hypersensitivity to the drug.
- ✓ Cellular response to the effects of cytokines is well regulated and it is reflected in the changes of gene expression in target cells resulting in the expression of new functions.

8.7 KEYWORDS

Growth Hormone, Cytokine, Tissue Plasminogen Activator and FactorVII

8.8 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Explain cytokines and its receptors
2. In detail mention the properties of cytokines
3. Comment on the structure & function of t-PA
4. Elaborate on the medical uses of Cytokine
5. Tissue Plasminogen Activator

Long Answer Questions

1. Describe the commercially important products with suitable examples
2. Comment on the production and medical uses of Cytokines
3. Discuss about the pharmaceutical and biotechnological uses of Growth Hormones

4. What is Factor VII &explain its role in normal blood clotting
5. In detail mention about the synthesis & secretion of t-PA & its mode of action

8.9 FURTHER READINGS

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UNIT-IX
MICROBIAL POLYSACCARIDES & POLYESTERS

Structure

9.1 INTRODUCTION

Microorganisms produce large amount of polysaccharides in the presence of surplus carbon source. Microbial polysaccharides are high molecular weight polymers of carbohydrate. They form the major component of cellular polymers found in and surrounding most microbial cells. Microbial polysaccharides are produced by a variety of microorganisms. They are water-soluble gums and have unique physical properties.

Polyesters are polymers formed from a dicarboxylic acid and a diol. They have many uses depending on how they have been produced and the resulting orientation of the polymer chains. The Polyhydroxyalkanoates or PHA**s** are polyesters produced in nature by numerous microorganisms including through bacterial fermentation of

sugar or lipids. This unit helps in understanding about the polysaccharides and polyesters from microorganisms.

9.2 OBJECTIVES

The objective of this chapter relies in furnishing details regarding Microbial Polysaccharides & Polyesters.

9.3 MICROBIAL POLYSACCARIDES

9.3.1 Definition and Structure

Microbial polysaccharides are either a part of the cell wall or excreted from the cell (extracellular polysaccharides) and are characterized as primary (e.g. several cell wall biopolymers) or secondary (e.g. several bacterial capsular biopolymers) metabolites.

Microbial polysaccharides are divided into three groups - exocellular, cell wall and intercellular. Exocellular polysaccharides are those that constantly diffuse into the cell culture medium and are easily isolated. The cell wall and intercellular polysaccharides are integral parts of the cell wall or capsular products. They are more difficult to separate from cell biomass. Microbial polysaccharides may be neutral or acidic in nature. Acidic polysaccharides which contains carboxyl group are commercially more important because they can function as polyelectrolytes.

Microbial polysaccharides are primarily linear molecules to which side chains of varying length and complexity are attached at regular intervals. Most microbial polysaccharides are linear hetero-polysaccharides consisting of three to seven different monosaccharides arranged in groups of 10 or less to form repeating units. The monosaccharides may be pentoses, hexoses, amino sugars or uronic acids. For example, xanthan gum is a polysaccharide produced by a pure-culture fermentation of a carbohydrate with *Xanthomonas campestris* and is composed of glucose, glucuronic acid, 6-acetylmannose and 4,6-pyruvylated mannose residues as seen in Figures 1 and 2.

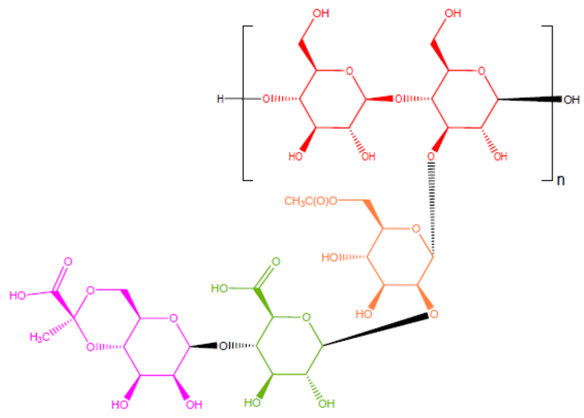


Fig.1 Xanthan Gum - a polysaccharide composed of glucose, glucuronic acid, 6-acetylmannose and 4,6-pyruvylated mannose.

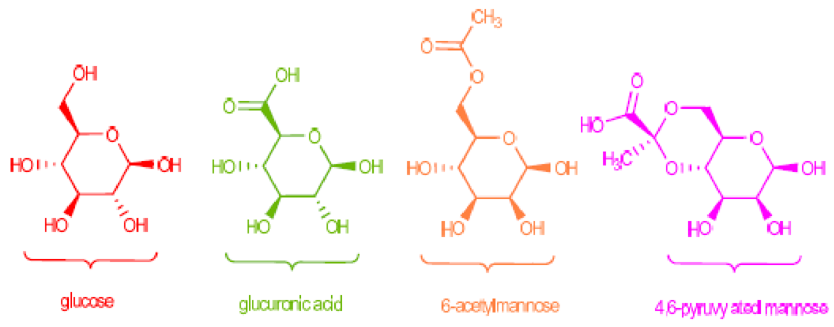


Fig.2 The monosaccharide components of Xanthan Gum - Glucose, glucuronic acid, 6-acetylmannose and 4,6-pyruvylated mannose.

9.3.2 Production

Microbial Polysaccharides are synthesized by providing excess source of carbon substrate and limiting the source of nitrogen supply. A ratio of 10:1 of carbon and nitrogen is considered more favourable for the optimal production of polysaccharides. The production process is mostly carried out by batch culture fermentation. By manipulating the nutrient supply in the medium, differential synthesis of polysaccharides can be obtained. Neutral polysaccharides can be produced by limiting the nitrogen supply. Acidic polysaccharides can be produced by limiting metal ions in the medium. Molecular oxygen supply of around 90% saturation is ideal for good growth and polysaccharide synthesis.

The pathways for the biosynthesis of polysaccharides are comparable to the formation of cell wall of the bacteria. More than 100

enzymatic reactions, directly or indirectly are involved in the synthesis of polysaccharides. Starting with glucose, appropriate sugars (by transforming glucose to others) are incorporated in the formation of polysaccharides. As the production of polysaccharides increases there will be an increase in the viscosity of the culture medium. The synthesized polysaccharides can be recovered by using appropriate techniques after appropriate solvents precipitate them.

9.3.3 Applications

Microbial polysaccharides have immense commercial and industrial applications. They are employed in the stabilization of foods and production of several industrial and pharmaceutical compounds. The commercial value of microbial polysaccharides is based on its rheological property (the ability to modify the flow characteristics of solutions). Polysaccharides are commonly used as thickening and gelling agents due to the property of increasing the viscosity. They are also used extensively in the oil industry. The major problem in the conventional extraction of oil is that nearly 50% of oil is found trapped in rocks or too viscous to be pumped out. Microbial enhanced oil recovery helps in the recovery of such viscous oils. It is done by injecting surfactants and viscosity decreasing microbial polysaccharides such as Xanthan and Emulsan.

Of the several microbial polysaccharides following are the selected lists of commercially important polysaccharides, the microorganisms used for the production, the method by which they are produced and the applications are given under Table 1.

Polysaccharide	Producing Microorganisms	Method	Applications
Xanthan	*Xanthomonas campestris*	Pure culture fermentation of glucose, recovered by precipitation and Purification by isopropyl alcohol, followed by drying and milling	Food addictive (Ice cream & Cheese) Oil Industry Preparation of Toothpaste and Water Based paints.
Gellan	*Pseudomonas elodea*	Aerobic submerged fermentation, Pure culture	In food industry as thickener and gelling

		fermentation of carbohydrate, recovered by precipitation with isopropyl alcohol, followed by drying and milling	agent
Dextran	*Leuconostoc mesenteroides* *Gluconobacter sp.,* *Streptococcus mutans*	Controlled hydrolysis and fractionation of polysaccharides elaborated by fermentation on sucrose	Blood plasma expander, used in the prevention of thrombosis & in wound dressing. In the lab of chromatographic and other techniques involved in purification, as a food stuff.
Scleroglucan	*Sclerotium glucanicum,* *Sclerotium rolfsii*	Pure culture fermentation	Used for stabilizing latex paints, painting inks and drilling muds
Pullulan	*Aureobasidium pullulans*	Fermentation of liquefied corn startch, Microfilteration, the filterate is heat sterilized and the pigments and other impurities are removed by adsorption and ion-exchange	Bio-degradable polysaccharide, used in food coating and packaging

		chromatography.	
Welan	*Alcaligenes* CGMCC2428, *Alcaligenes sp.* (ATCC 31555)	Fermentative production	Cement manufacturing as a thickening agent, emulsifier
Alginate	*Pseudomonas aeruginosa, Azobactervinelandii*	Pure culture Fermentation	Food industry as thickening and gelling agent, Alginate beads are employed in immobilization of cells and enzymes, Used as ion-exchange agent
Curdlan	*Alcaligenes faecalis*	Pure culture Fermentation	As a gelling agent in cooked foods, Useful for immobilization of enymes
Emulsan	*Acinetobacter calcoaceticus, Arthrobacter sp.*	Pure culture Fermentation	In oil industry for enhanced oil recovery, For cleaning of oil spills

Table 1 Methods of Production and Applications of Polysaccharides from Microorganisms

9.3.4 Xanthan

Xanthan gum is a natural polysaccharide and an important industrial biopolymer. It was discovered in the 1950s at the Northern Regional Research Laboratories (NRRL) of the United States Department of Agriculture. Xanthan is an acidic polymer made up of pentasaccharide subunits forming cellulose backbone withtrisaccharide

side-chains. Two glucose units, two mannose units and one glucuronic acid unit in the molar ratio of 2.8:2:2 form these pentasaccharide units.

The backbone of the polysaccharide chain consists of two D-glucose units linked through the 1 and 4 positions but the unique character of xanthan gum is derived from the trisaccharide side chain on alternate sugar units. The molecular weight of xanthan varies in distribution ranging from $2x10^6$ to $20x10^6$ Da. This molecular weight distribution depends on the association between chains forming aggregates of several individual chains. The variations of the fermentation conditions used in production are factors that can influence the molecular weight of xanthan.

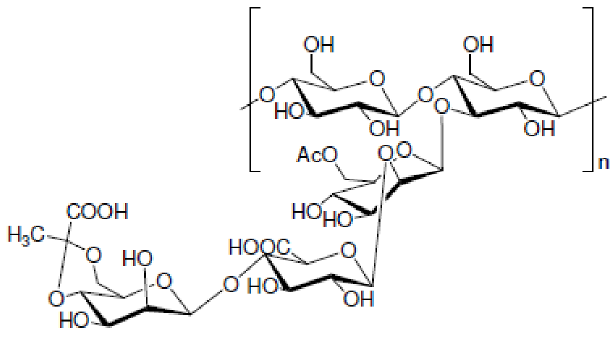


Fig. 3 Chemical Structure of Xanthan

9.3.5 Gellan

Gellan is a bacterial exopolysaccharide produced by aerobic submerged fermentation of *Sphingomonaspauci mobilis* (previously called *Pseudomonas elodea*). This linear anionic heteropolysaccharide of 500 kDa possesses a trisaccharidic repeating unit - [,3)-b-D-Glc-(1,4)-b-D-GlcA-(1,4)-b-D-Glc-(1,4)-a-L-Rha-(1,]n , substituted by acyl groups such as acetate at C6 and L-glycerate at C2 on the linked D-glucose unit adjacent to the glucuronic acid unit (1 mole of glycerate and 0.5 mole of acetate per repeat unit).

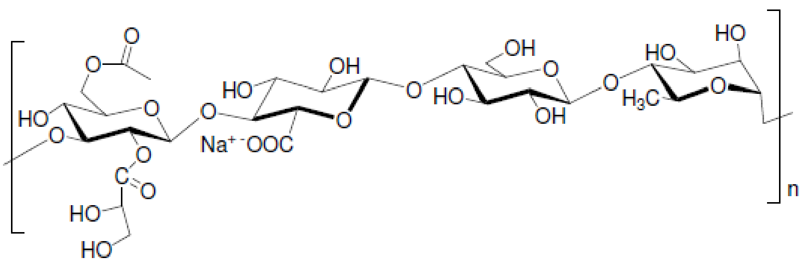


Fig. 4 Chemical Structure of Gellan

9.3.6 Curdlan

Curdlan is a neutral gel-forming b-(1,3)-D-glucan. It was first detected in *Agrobacterium* biovar. 1 (formerly *Alcaligenesfaecalis var. Myxogenes* strain 10C3), co-produced with another extracellular polysaccharide: the succinoglycan, an acidicheteroglycan. It consists of as many as 12,000 glucose units linked by b-glucosidic bonds at C1 and C3 [,3)-b-D-Glc*p*-(1,]. It was reported that the number-average molecular weight of bacterial curdlan is in the range of 5.3×10^{4} Da to 2.0×10^{6} Da. Recently, the molecular weight of curdlan produced by the mutant strain of *Agrobacterium* sp. ATCC 31750 was about 300,000Da.

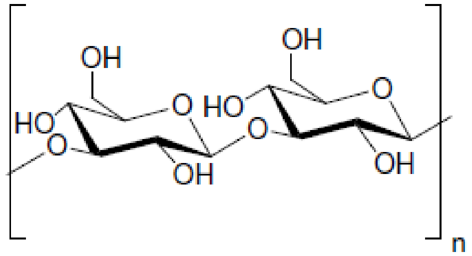


Fig. 5 Chemical Structure of Curdlan

9.4 POLYESTERS

9.4.1 Definition and Structure

The Polyhydroxyalkanoates or PHAs are polyesters produced in nature by numerous microorganisms including through bacterial fermentation of sugar or lipids. Different microorganisms were found to accumulate various PHAs comprising (R)-hydroxycarboxylic acids having a carboxyl group at the end and a hydroxyl group at 3-,4-,5 or 6-position; to date, more than 150 kinds of hydroxycarboxylic acids have been found as the monomers of PHAs. They serve as a source of energy and as carbon storage. These plastics are biodegradable making them environmental friendly and are used in the production of bioplastics.

PHAs are categorized into two groups depending on the total number of carbons in the monomer 1) short-chain-length (SCL)-PHAs having 3 to 5 carbon atoms and 2) medium-chain-length (MCL)-PHAs having 6 to14 carbon atoms. SCL-PHAs show thermoplastic material properties similar to polypropylene while MCL-PHAs possess elastic material properties similar to rubber. Interestingly, some microorganisms synthesize PHAs having both SCL and MCL-

monomers. Such SCL-MCL-PHAs exhibit material properties similar to low density polyethylene.

9.4.2 Production

PHAs can be produced by fermentation of microorganisms whether they are natural isolates or synthesized. Industrial PHA production processes have been developed for poly(3 hydroxybutyrate) (poly(3HB)) and poly(3-hydroxybutyrate-*co*-3-valerate) (poly(3HB-*co*-3HV).Many bacteria have been screened to produce poly(3HB) or poly(3HB-*co*-3HV)(a copolymer consisting of 3-hydroxybutyrate and 3-hydroxyvalerate). The overall general microbial production of polyesters is given below.

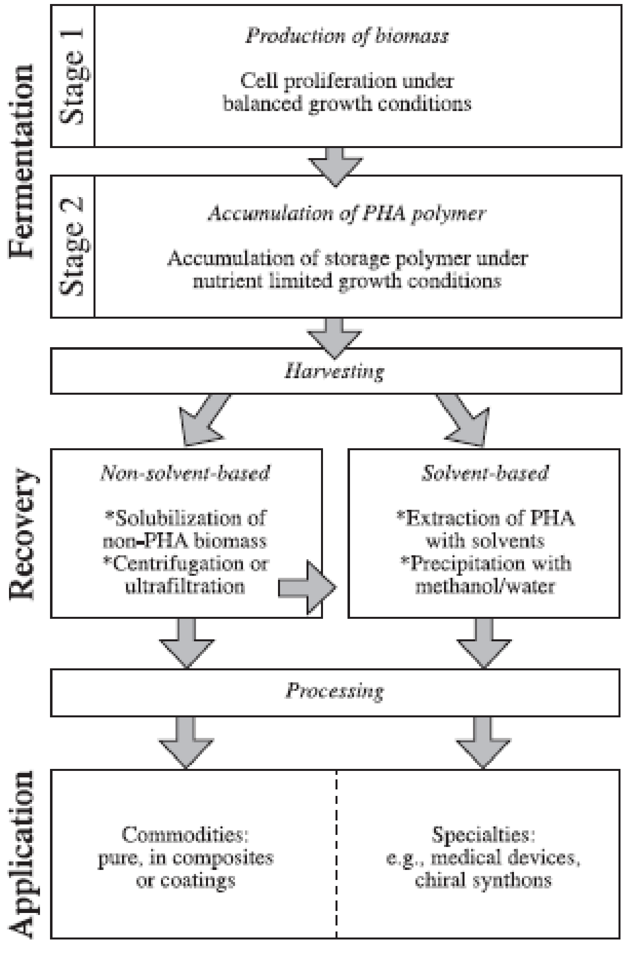


Fig. 6 General Overview of PHA production Process

9.4.3 Poly (3HB) and Poly (3HB-co-3HV)

The suitability of a bacterium for poly(3HB) production on an industrial scale depends on many different factors such as stability, achievable cell densities and poly(3HB) contents, extractability of the polymer, molecular weights of accumulated poly(3HB), range of utilizable carbon sources, costs of the carbon source and occurrence of by-products.

The fermentation is a two-step fed batch process. The process utilized a mutant of *Ralstonia eutropha* (formerly called *Alcaligenes eutrophus*). During the first step, the cells were grown in a mineral salt medium with glucose as sole carbon and energy source and a calculated amount of phosphate based on the known requirements of the organism to allow production of a given amount of biomass. As the culture grew, phosphate was depleted from the medium and during the second step, when phosphate was limiting, the cells start to produce and store polymer. Glucose was then added to the culture and the fermentation was continued until the required polymer content was reached. The copolymer of poly (3HB-*co*-3HV) was made by providing a mixed feed of glucose and propionic acid in the polymer accumulation phase.

9.4.4 Poly (3HAMCL)s other than Poly (3HB) and Poly (3HB-*co*-3HV)

Fluorescent *Pseudomonads* are capable of synthesizing poly (3HAMCL)s from a large number of substrates. *Pseudomonas oleovorans* and *P. Putida* are used in the production of poly (3HAMCL). *P. oleovorans*is able to use alkanes and alkenes as substrate due to the presence of the OCT-plasmid. *P. putida* can use carbohydrates such as glucose and fructose for the production of poly (3HAMCL).

P. oleovorans was grown in two liquid phase fed-batch cultures. The two phases consisted ofa watery phase containing mineral nutrients and an organic phase of octane. Using an organic phase is convenient because this results without extra addition during the process, in a constant availability of the carbon source for the microorganisms in the watery phase. Biomass was produced in the first phase, while in the second stage poly (3HAMCL) was synthesized in the absence of a nitrogen source.

Poly (3HAMCL) production processes with *P. putida* have been developed in parallel. In contrast to *P. oleovorans*, *P. putida* does not have to be grown under nutrient-limited conditions in the presence of a carbon excess to produce poly (3HAMCL). *P. putida* uses fatty acids rather than alkanes or alkenes as substrate for poly (3HAMCL)

formation. These fatty acids however cannot be used as a second phase during fermentation because the resulting high fatty acid concentrations are toxic to *P. putida*. A method has been developed in which discrete pulses of fatty acids were added to fed-batch cultures. Substrate exhaustion was detected by a sudden increase in dissolved oxygen tension and this signal was used to trigger the injection of another fatty acid pulse into the fermentor. This minimized the time during which the culture was carbon limited, while the maximum concentration of fatty acids could be kept below toxic levels.

9.4.5 Applications

Poly (3HAMCL) is a family of many different polymers which can all be produced using the same or similar fermentation processes by simply changing the type of substrate(s) used. This enables the production of tailor made poly (3HAMCL) variants for specific applications. The production of bacterial polyesters came from a combination of the availability of large-scale fermentation equipment, the instability of the oil market, looming oil shortages and growing interest in biodegradability and sustainability. Specialty applications of PHAs include hydrophobic coatings, specialty elastomers, medical implants and functionalized polymers for chromatography, microgranules to be used as binders in paints or in blends that incorporate latexes and as sources of chiral monomers.

9.4 CHECK YOUR PROGRESS QUESTIONS

1. What is Xanthan?
2. Name the microorganism that produces Alginate
3. What is PHA?
4. Name the microorganisms that produce Poly (3HAMCL)

9.5 ANSWERS TO CHECK YOUR PROGRESS

1. Xanthan is a natural polysaccharide made up of glucose, glucuronic acid, 6-acetylmannose and 4,6-pyruvylated mannose.
2. *Pseudomonas aeruginosa, Azobacter vinelandii*
3. Polyhydroxyalkanoates
4. *Pseudomonas oleovorans, Pseudomonas putida*

9.6 SUMMARY

In this unit, you have learnt about the meaning, need and objectives of the microbial polysaccharides, production of polysaccharides and its application with examples of microbial polysaccharides include Xanthan, Gellan and Curdlan. This unit also

NOTES

talks about the polyesters, microbial production of polyesters such as Polyhydroxyalkanoates, their related derivatives and their industrial applications.

9.7 KEYWORDS

Microbial Polysaccharides, Xanthan, Gellan, Curdlan, Polyhydroxyalkanoates, Poly(3HB) and Poly(3HB-co-3HV).

9.8 SELF ASSESSMENT QUESTIONS & EXCERCISES

1. Define Microbial polysaccharides with the structure as an example
2. Write down any five polysaccharides, their source organism for production and its applications
3. What is PHA? Explain its industrial production
4. Comment on the applications of PHAs

9.9 FURTHER READINGS

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UNIT-X
BIOCOMPOST, BIOGAS & MICROBIAL FUEL CELL

Structure

10.1 INTRODUCTION

Bio-composting is a process which converts the organic wastes into humus with the help of mesophilic and thermophilic organisms. It is an economical method to recycle organic wastes with the help of biological agents and an effective method for recovery of nutrients trapped in organic waste materials. The process of composting seeks to connect the natural forces of decomposition and conversion of organic wastes into organic fertilizer.

The composting of agricultural wastes and municipal solid wastes (MSW) has a long history and is commonly used to recycle organic matter back into the soil to maintain soil fertility. Composting is seen as an environmentally acceptable waste treatment method. Both aerobic and anaerobic biological process uses naturally occurring microorganisms to convert biodegradable organic matter into humus like product. The process destroys pathogens, converts N from unstable ammonia to stable organic forms, reduces the volume of waste and improves the nature of the waste. It also makes wastes easier to handle and transport, often allows for higher application rates because of the more stable, slow release in nature of the N in compost. Bio fertilizer made by composting process has been identified as an alternative to chemical fertilizer to enhance soil fertility and crop production.

Composting is a biological process which converts heterogeneous organic wastes into humus like substances by mixed microbial population under controlled optimum conditions of moisture, temperature and aeration. It is the aspect of control that separates composting from natural rotting or decomposition processes which occur in an open dump, sanitary landfill or unmanaged waste pile. In composting, microorganisms convert organic materials such as manure, **sludge, leaves, fruits, vegetables and food wastes into product like soil** humus. Through composting, organic wastes materials are decomposed and stabilized into a product that can be used as soil conditioner and/or organic fertilizer. Decomposers include bacteria, actinomycetes and fungi that are widespread in nature. These are indigenous to soil, dust, fruit and vegetable matter and waste of all sorts, so special organisms are not required. Controlled decomposition occurs as a result of activities of these naturally occurring microorganisms.

Composting can be considered as microbial farming, so they need energy, food and habitat. These microorganisms require carbon as energy source and nitrogen to build proteins. Bacteria produce enzymes to break down complex carbohydrates into simpler forms and use them as food. Composting process continues until the remaining nutrients are consumed by the last microorganisms and most of the carbon is converted into carbon dioxide and water. The nutrients that become available during decomposition remain in the compost within the bodies of dead microorganisms and in humus.

There are two main groups of organisms which decompose organic matter:

1. Anaerobic bacteria which perform their work in the absence of oxygen.

2. Aerobic bacteria which perform their work in the presence of oxygen.

10.2 OBJECTIVES

The objective of this chapter relies in furnishing details regarding The production and application of Biocompost , Biogas and Microbial Fuel Cell.

10.3 CLASSIFICATION OF SOLID WASTES

Solid wastes can be broadly categorized into five broad categories depending on their origin and its intended use:

a) Biodegradable Wastes

Food and kitchen wastes, green wastes (vegetables, flowers, leaves fruits), paper.

b) Recyclable Material Wastes

Paper, glass, bottles, cans, metals, certain plastics.

c) Inert Wastes

Construction and demolition wastes, dirt, rocks, debris.

d) Composite Wastes

Waste clothing, tetra packs and waste plastics such as toys.

e) Domestic hazardous waste and toxic waste

Medication, e-waste, paints, chemicals, light bulbs, fluorescent tubes, spray cans, fertilizer and pesticide containers, shoe polish.

Can be composted	Cannot be composted
Sewage sludges	Coal ash.
Industrial wastes (e.g. food, pulp & paper).	Metal, glass and plastic.
Yard and garden wastes.	Nappies.
Municipal solid wastes (up to 70% organic matter by weight).	The roots of persistent weeds, like bindweed and couch grass.
Soft prunings, clippings and leaves	Leaves with persistent disease such asblackspot.
Kitchen waste like fruit, peelings, teabag sand egg shell.	Meat or fish.
Paper shredded, mixed with grass cuttings and used sparingly.	Cooked food, especially meat, as this attracts vermin.

Table 1. Types of wastes that are suitable and unsuitable for bio-composting

10.4 TYPES OF COMPOSTING

Composting is a controlled aerobic process to decompose organic materials that utilizes microbes in preferably thermophilic temperature conditions (40-65°C). During the process, microbes consume oxygen and release heat, CO_2 and water vapor. Consequently, significant reduction in weight and volume of the waste is obtained.

$$\text{Organic compounds} + O_2 \xrightarrow[\text{microbes}]{\text{Temperature} = 40\text{ - }65°C} CO_2 + H_2O + \text{Heat}$$

Generally, the composting process starts with the decomposition of easily degradable materials into stabilized material in the presence of oxygen. This leads to the release of heat and subsequent rise in

temperature of the compostable material. This situation sustains for several weeks until faster decomposition of organic matter takes place. Once all easily biodegradable organic compounds are consumed, temperature of the feedstock is dropped to the ambient temperature. This period is followed by curing period that is characterized by slow decomposition of the material. Almost no further reduction in weight indicates the end of the curing period. The whole process takes around 20-30 days to complete.

Anaerobic composting is a long process (4 to 12 months) and takes place at low temperature, thus the destruction of pathogens is not fully accomplished. As a result, nutrients are lost and gaseous products of reduction like methane, hydrogen sulphide are produced which have offensive odors. Aerobic composting is characterized by rapid decomposition (8-10 weeks). During this period, high temperature are attained (exothermic reaction) leading to speedy destruction of pathogens, insect eggs and weed seeds. The production of foul-smelling gases like methane, hydrogen sulphide is minimized and nutrients are fairly preserved. The effectiveness of the composting process is influenced by factors such as temperature, oxygen supply and humidity content.

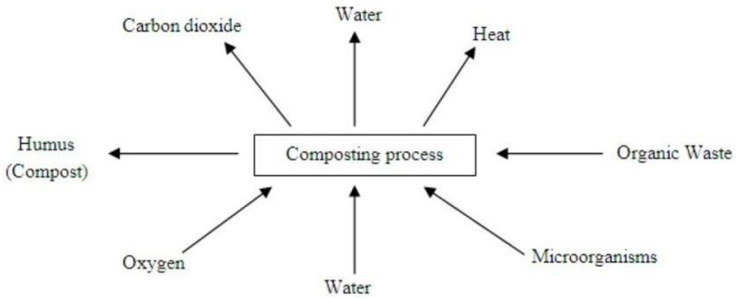


Fig 1 An Overview of the Bio-composting Process

Aerobic Composting

Composting is the decomposition of organic wastes in the presence of oxygen; the process includes CO_2, NH_3, water and heat. This can be used to treat any type of organic waste but, effective composting requires the right combination of ingredients and conditions. These include the moisture contents around 60-70% and Carbon to Nitrogen (C / N) ratios of 30:1. Any significant variation inhibits degradation process. In general, wood and paper provide an important source of carbon, while sewage sludge and food waste provide nitrogen to ensure an adequate supply of oxygen at all times. Ventilation of waste, either forced or passive is essential.

Anaerobic Composting

Anaerobic Composting is the decomposition of organic wastes in the absence of O_2, the products being methane (CH_4), CO_2, NH_3 and trace amounts of other gases and organic acids. Anaerobic composting was traditionally used to compost animal manure and human sewage sludge, but recently it is more common for some municipal solid wastes (MSW) and green wastes to be treated in this way.

10.5 IMPORTANT PARAMETERS OF COMPOSTING PROCESS

Microorganisms

Microorganisms decompose or oxidize the organic compounds to simple, stabilized end products with the production of heat. During the process, oxygen is consumed and carbon dioxide, water and often ammonia are released. The heat energy is partially used for cell synthesis of the microorganisms. However, the heat production is sufficient to raise the temperature up to the thermophilic range. The shape of the temperature curve depends on the initial waste materials being composted and the composting methods. However, in a controlled reactor system the variation in temperature distribution can be improved. Biological waste materials contain a large number of many different types of bacteria, fungi, mold and other living organisms. High-temperature compost appears to be a promising source for isolation of new thermophilic organisms as well as heat-stable enzymes of industrial value. More species of bacteria are involved in aerobic decomposition than in anaerobic fermentation. The main microorganisms responsible for biological degradation in composting are bacteria, actinomycetes and fungi of mesophilic and thermophilic groups.

Types	Species
Bacteria	*Bacillus casei,*
	Lactobacillus buchneri
Fungi	*Candida rugopelliculosa*
	Trichoderma
	White-rot fungi
Bacteria	*Pseudomonas*
	Azotobacter
	Azospirillum Micrococcus
Actinomycetes	*Streptomyces*
	Actinomyces
Fungi	*Trichoderma*
	Alternaria
	Penicillium and Aspergillus

Table 2. Most commonly used microorganisms for Solid Waste Composting

Aeration

Composting systems are distinguished on the basis of oxygen usage (aerobic/anaerobic). Aerobic decomposition, in contrast to anaerobic types, is quicker, progresses at higher temperatures and does not produce foul odors. While anaerobic decomposition may be conducted with minimal operator attention and the operation may be sealed from the environment. However, the most modern composting operations attempt to maintain an aerobic environment. Mixing the compost pile at intervals, aerates it, but it is often difficult to determine the exact periods to turn the pile. Aeration conducted in excess is usually not harmful to the composting process, except that an optimum temperature is harder to maintain and excessive evaporation may cause moisture to become a limiting factor. An oxygen level from 10 to 30% has been found optimum for the process. The air supply needed for temperature and moisture control typically is ten or more times greater than those for biological decomposition, so that when these needs are met, biological oxygen demands also will be safely satisfied. Several systems have been applied to provide aeration for composting. The main systems are mechanical turning and forced aeration via air blower or fan. Forced aeration composting is usually applied to static windrow, static pile and to most of the reactor systems. To control the rate of aeration, a number of strategies have been designed and practiced for the composting process. These range from simple manual control systems to more sophisticated computer control system using temperature, oxygen or carbon dioxide feedback as the controlling variables and air supply as the manipulated variable.

Carbon/Nitrogen Ratio

The carbon to nitrogen (C/N) ratio is one of the important factors affecting the composting process as well as the properties of the end product. A C/N ratio between 25 and 30 is usually considered as the optimum ratio for composting. During composting, microorganisms utilize the C as a source of energy and the N for building cell structure. Microorganisms utilize C and N at a ratio of about 30:1. High or low C/N ratios can be adjusted by adding high nitrogen or carbon rich wastes respectively. Sawdust, wheat straw, grass clippings, dry leaves are examples of carbon-rich materials, whereas poultry manure, slaughterhouse waste, sewage sludge. are nitrogen-rich. The C/N ratio decreases during the composting process as carbon is lost in the form of carbon dioxide.

Temperature

Rate of organic matter decomposition depends on the temperature of the raw material. The decomposition of organic compounds starts in a temperature range (40-65°C). Temperatures higher than 50°C should be maintained for at least 3-4 days to destruct the harmful organisms such as plant pathogens, weed seeds and fly larvae.While low temperatures retard composting, and may even halt the process. Low temperatures are indicative of reduced microbial activity and could indicate a lack of oxygen or inadequate moisture conditions. During the initial stages of composting, the active microbial population grows exponentially until the available substrate or other factors limit growth. It is reported that temperature as high as 85°C doubles the decomposition rate than at 55°C. However, this high temperature is fatal for a certain microbial population. Hence, most of the modern composting plants are operating in thermophilic temperature range (55-65°C).

pH

Metabolic activities affect the pH of compost under process. Efficient biological decomposition of organic wastes depends on optimum pH (6.0-7.5). Deamination of protein rapidly increases the pH due to ammonia. Conversely, production of organic acids during the decomposition of carbohydrates and lipids decrease the pH. On average, pH of inputs is somewhat acidic while finished compost is neutral. Considerable changes in pH value occur during the composting process. In the beginning, the formation of organic acids and carbon dioxide lower the pH value to approximately 5.0 or less, whereas as the process progress, the pH value reaches up to 8.0 to 8.5. The pH of compostable material influences the type of organisms involved in the

composting process. Fungi tolerate a wider pH range than bacteria do. The optimum pH range for most bacteria is between 6.0 and 7.5, whereas for fungi it can be between 5.5 and 8.0. Most of the waste materials available for composting are within the above pH range and hence pose no problem of pH control.

Moisture

Moisture content is very important for composting and it may become the limiting factor if not monitored. Excess water interferes oxygen accessibility, while too little hinders diffusion of soluble molecules and microbial activity slowing down the rate of composting. Moisture content of 40 to 60% has been found optimum for good composting process. While a moist mixture is necessary to sustain the biological decomposition vital to the composting process, dry compost is easier to manipulate and store without causing a nuisance. Only after composting has been completed, drying could be considered as a necessary prerequisite for storage or sale.

Particle Size

Particles size affects oxygen movement into the pile as well as microbial and enzymatic access to the substrate. Smaller size particles of organic material increase the surface area available for microbial attack. However, very small particles pack tightly together; preventing movement of air into the composting heap and movement of carbon dioxide out of the heap. Large size particles reduce the surface area for microbial attack which slows down or may stop composting process altogether. Bulky organic materials should be chopped or shredded to reduce particle size to the range of 1-5 cm. On the other hand, if too small, the organic materials should be mixed with a bulking agent like wood chips or tree bark. A particle size of 5 cm is appropriate for heaps employed to natural air flow, while 10 mm size is suitable for the composting systems having forced air supply.

Time

Total organic matter decomposition period depends on a number of factors such as nature of the contaminants, temperature, oxygen availability, particle size, moisture content. For example, generally period of active composting for dairy cattle waste is around 10-14 weeks. This stage is followed by 3-4 weeks curing period.

Stages of Composting

A large variety of mesophilic, thermotolerant and thermophilic aerobic microorganisms predominantly bacteria, actinomycetes, yeasts and fungi are involved in the specialized biodegradation process. The process of bio-composting occurs inthree phases:

(a) Mesophilic Phase

(b) Thermophilic Phase, which can last from a few days to several months

(c) Cooling and Maturation Phase which lasts for several months.

The length of the composting phases depends on the nature of the organic matter being composted and the efficiency of the process which is determined by the degree of aeration and agitation. At the start of composting, the mass is at its ambient temperature and usually slightly acidic. Soluble and easily degradable carbon sources, monosaccharides, starch and lipids are utilized by microorganisms in the early stage of composting. The pH decreases because organic acids are formed from these compounds during degradation. In the next stage, microorganisms start to degrade proteins, resulting in the liberation of ammonia and increase in the pH. As the temperature increases, thermophilic microbes develop. These consists of only a few genera of bacteria e.g. *Bacillus subtilis*, fungi e.g. *Aspergillus fumigatus*, and actinomycetes e.g. *Streptomyces sp*. After the easily degradable carbon sources have been consumed, more resistant compounds such as cellulose, hemicellulose and lignin are degraded and transformed into humic acid, fulvic acid and phenolic intermediate metabolites.

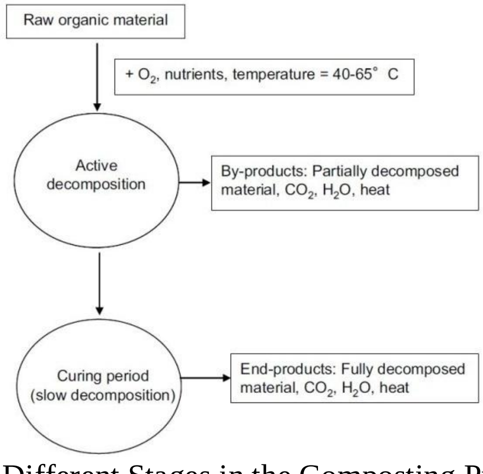


Fig 2. Different Stages in the Composting Process

10.6 Methods of Composting

Open Windrows

This is the least sophisticated technique which involves placing a mixture of organic waste materials into long, narrow piles approximately six feet high by twelve feet wide and as long as it is

necessary. The composting process depends upon a good supply of oxygen, therefore air must be able to move through the windrow. This will depend upon the size and shape of the windrow, the porosity of the material and its water content. Feedstock material is usually shredded to ensure the correct porosity. A windrow constructed of low-density materials such as leaves can be much larger than a windrow constructed of wet dense manure. Anaerobic areas can occur near the center of the windrow if it is too large, too dense or too wet and these areas will release odors when the windrow is turned. On the other hand, small windrows lose heat quickly and may not achieve high enough temperatures to kill pathogens and weed seeds. Turning releases trapped heat, water vapour and gases and also mixes the materials, breaks up large particles and restores the pore spaces eliminated by decomposition and settling. Turning also exchanges the material from the outside of the windrow with that from the interior. This helps to ensure that all material receives equal exposure to the air at the surface and to the high temperatures inside the windrow thereby providing a uniform treatment process. The compost process may take 4 to 6 weeks and finally the compost is ready to use as a fertilizer.

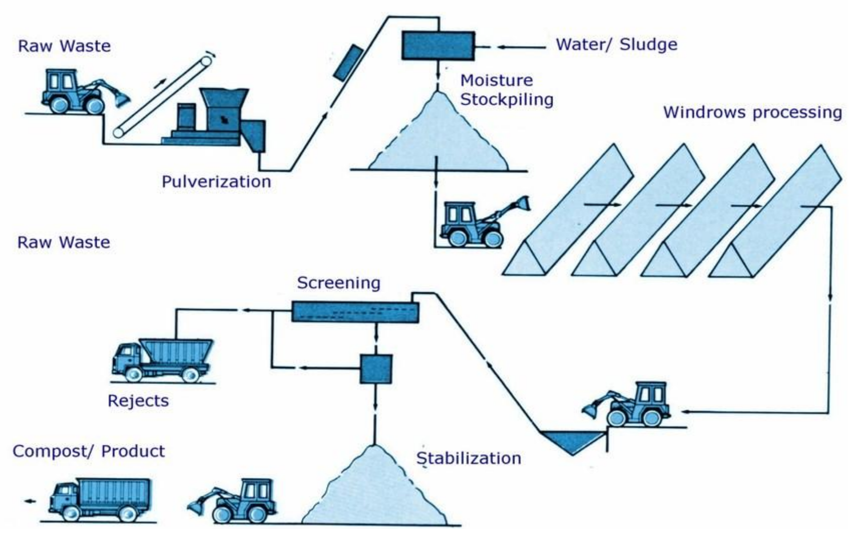


Fig 3. Windrow CompostingProcess

Aerated Static Pile

This system involves supply of ambient air through mechanical means and requires no turning of the organic mixture once the pile is formed. By controlling air mechanically, this process allows the use of larger piles. For composting under this method, an air plenum is constructed and the organic mixture is placed in piles on top of the air plenum. Piles are built as high as the equipment allows (Normally it is kept eight to twelve feet high). Aerated static piles can be constructed individually or in extended piles. Individual piles, constructed all

atonce, allow the composting to occur in batches. Extended piles consists of a series of cells created over the course of many days and stacked against each other to form one long rectangular pile. A temperature sensor placed within the pile works in conjunction with the blower to control temperature and oxygen concentration within the pile.

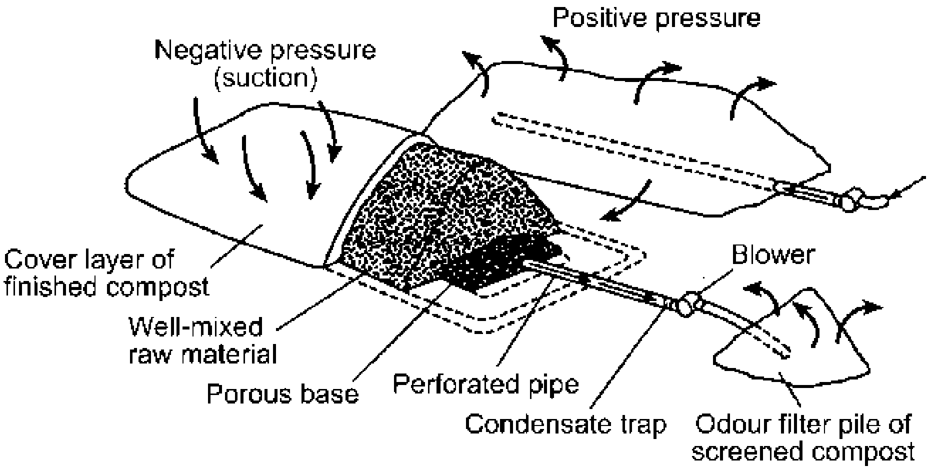


Fig 4. Bio-composting by Aerated Static Pile (Negative and Positive Pressure)

In-vessel Composting

It involves confining the compost process to a variety of containers or vessels. Different in-vessel systems use a variety of methods to accelerate the composting process. These systems usually include provisions for aeration, mixing, temperature control and containment of odors. In-vessel systems generally are the costliest of the three major technologies because of its high construction costs. It is designed to accelerate the decomposition process of organic materials andthe management of these processes will either speed up or slow down the decomposition process ultimately influencing the quality and cost of the product.

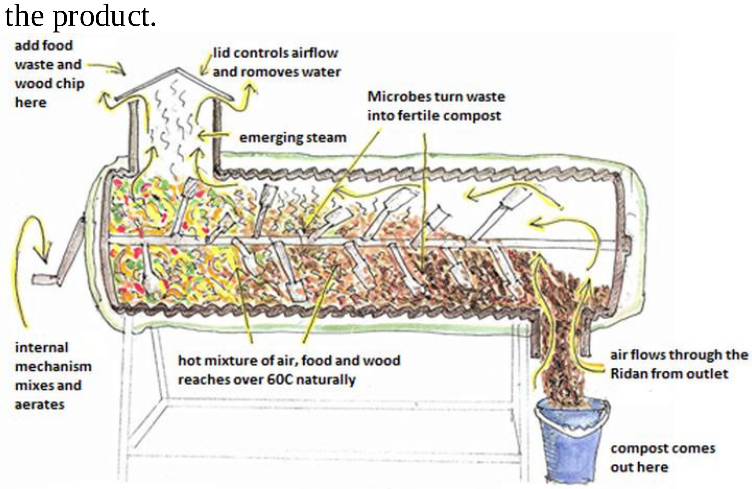


Fig 5. Bio-composting Process through In-vessel Composting

Vermicomposting

Vermicomposting is the result of combined activity of microorganisms and earthworms. Earthworms will break down sewage sludge and other organic wastes. The 'tiger' worm (*Eisenia fetida*)grows well in a wide range of wastes including pig and cattle solid and slurries, horse manure and potato waste. Worms are useful in converting agricultural wastes into useful soil conditioners, but also the worms can be harvested and processed into a nutritious protein feed supplement for fish, poultry and pigs. *E. fetida*prefers a pH of 5, temperatures <35ºC and it will not enter poultry wastes with high ammonia content. The amount of soluble P, K and Mg increases and worm-processed animal wastes have been shown to be suitable as plant growing media.

	Composting System		
	Windrow	ASP	In-Vessel
Preferable waste input	All type of wastes, but preferable for those with less emission of odour such as the plant-based wastes	Preferable for waste with more homogeneity and consistency and bulking agent is required	All type of wastes but preferable for easily degraded wastes such as food waste
Loading capacity	Can accommodate > 10 t of waste. As long as the rise in temperature is observed and maintained for at least 3 d, the functionality of the system is ensured. Usually, the compost pile is more than 1 (Length, L) × 1 (Width, W) × 1 (Height, H) m	Can accommodate > 10 t of waste. As long as the rise in temperature is observed and maintained for at least 3 d, the functionality of the system is ensured. Usually, the compost pile is more than 1 (L) × 1 (W) × 1 (H) m	Usually, can accommodate around 1 – 5 t of waste for the whole composting process
Land area requirement Site selection and transportation of waste	High The site has to be away from populated area, thus higher waste transportation cost	Medium The site has to be away from populated area, thus higher waste transportation cost	Low The site can be anywhere that can accommodate the composter, and a site nearer to the waste source can be selected
Composting period	Long Faster than passive ASP but slower than active ASP	Long ASP with active airflow will give higher efficiency compare to passive ASP	Short
Type of amendment can be considered	Increase aeration and addition of bulking agent, chemical additive, and microbial additive	Increase airflow in active ASP and addition of bulking agent, chemical additive, and microbial additive	Usually in mechanical aspect, increase the system temperature, pressure, and turning frequency
Composting period (with amendment)	Can be reduced by more than 30 % if amendment successfully applied	Beside increase airflow which might give a similar efficiency than windrow, effect of the rest of the amendments will be lower than windrow system	> 50 % of the time in composting can be reduced, but the curing phase still takes around 4 – 8 weeks.
Compost quality	Medium to good	Medium to good	Good

Table 3 Comparisonof different bio-composting techniques (Windrow, Aerated Static Pile and In-Vessel)

10.7 Benefits of Bio-composting

Soil

- Improves its physical structure.
- Enriches soil with microorganisms.
- Microbial activity in worm castings is 10 to 20 times higher than in the soil and organic matter that the worm ingests.
- Attracts deep-burrowing earthworms already present in the soil.
- Improves water holding capacity.

Plant Growth

- Enhances germination, plant growth and crop yield.
- Improves root growth and structure.
- Enriches soil with microorganisms.

Economic

- Biowastes conversion reduces waste flow to landfills.
- Elimination of bio-wastes from the waste stream reduces contamination of other recyclables collected in a single bin.
- Creates low-skill jobs at rural level.
- Low capital investment and relatively simple technologies make vermicomposting practical for less-developed agricultural regions.

Environmental

- Helps to close the "metabolic gap" through recycling waste on-site.
- Large systems often use temperature control and mechanized harvesting, however other equipment is relatively simple and does not wear out quickly.
- Production reduces greenhouse gas emissions such as methane and nitric oxide.

10.8 Biogas

Biogas typically refers to a mixture of different gases produced by the breakdown of organic matter in the absence of oxygen. Biogas can be produced from raw materials such as agricultural wastes, manure, municipal wastes, plant materials, sewage, green wastes or food wastes. Biogas is a renewable energy source and, in many cases, exerts a very small carbon footprint. Biogas can be produced by anaerobic digestion with anaerobic organisms which digest material

inside a closed systemor fermentation of biodegradable materials. Biogas is primarily methane and carbon dioxide and may have small amounts of hydrogen sulphide, moisture and siloxanes. The gases methane, hydrogen and carbon monoxide can be combusted or oxidized with oxygen. This energy release allows biogas to beused as a fuel; it can be used for any heating purpose such as cooking. It can also be used in a gas engine to convert the energy in the gas into electricity and heat. Biogas can be compressed, the same way natural gas is compressed to CNG and used to power motor vehicles. Biogas is considered to be a renewable resource because its production-and-use cycle is continuous and it generates no net carbon dioxide. Organic material growth is converted and used and then regrows in a continually repeating cycle. From a carbon perspective as much carbon dioxide is absorbed from the atmosphere in the growthof the primary bio-resource as is released when the material is ultimately converted to energy.

Biomass Sources

Historically, humans have harnessed biomass-derived energy sincethe time when people began burning wood to make fire.Even today, biomass is the only source of fuel for domestic use inmany developing countries. Biomass is all biologically-producedmatter based in carbon, hydrogen and oxygen.Wood energy is derived by using lignocellulosic as fuel. Harvested wood may beused directly as a fuel or collected from wood waste streamsto be processed intopellets. The largest source of energy from wood is pulpingliquor or 'black liquor', a waste product from processingof the pulp, paper and paperboard industry.Biomass including plant or animal matterthat can be converted into bio fuels.

Typical composition of biogas		
Compound		%
Methane		50–75
Carbon dioxide		25–50
Nitrogen		0–10
Hydrogen		0–1
Hydrogen sulphide		0–3
Oxygen		0–0.5

Table 4. Composition of Biogas

Biogas Generation

Today, there are several different techniques for producing biogas and several models and designs of biogas machines and plants now exist. Nevertheless, the concept remains simple and the same. The heart of any biogas system or production arrangement is known as a biodigester. A digester is a sealed and airtight tank or container that

behaves like the stomach of a human being. It collects wastes and digests it with the help of bacteria. The digestion must happen in the absence of oxygen for the production of Biogas. The valuable by product of this digestion process is methane. The methane gas that is produced usually rises and builds up at the top of the digester. A gas pipe is attached to the top of the digester to carry the produced gas back into the house where it is used as fuel for cooking and heating.

Other parts of a biogas system include a feeder pipe through which the waste materials enter the digester. There is also an overflow pipe or tank that ensures that the pressure inside the digester remains within safe limits. The final other interesting part of a biogas plant is an outlet or collection pipe for the solid and liquid residue that is left behind after the digestion process. This residue is commonly referred to as a 'biofertilizer' because it is very rich in nitrogen and phosphorus which makes it a good manure/fertilizer for your garden or small farm. Biogas production has a long-life span. A small-scale biogas production plant in your backyard can last for up to 20 years and will require little maintenance during its life time.

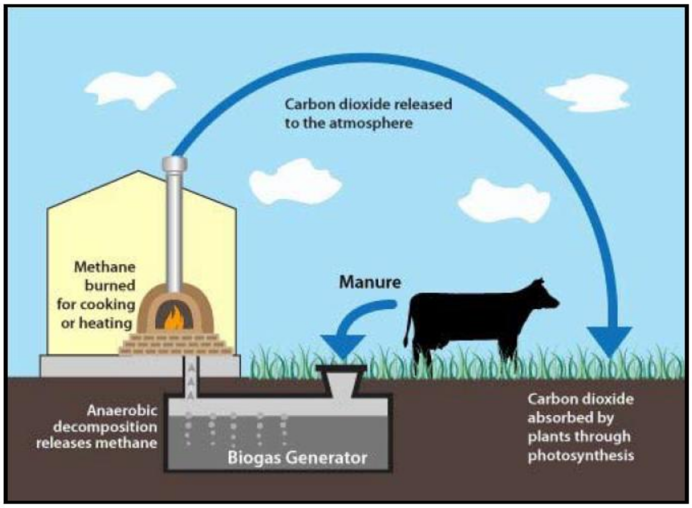


Fig 6. Generation of Biogas by Traditional Method

Gas Production Figures

If the daily amount of availabledung (fresh weight) is known, gas production per day in warmtropical countries will approximately correspond to the followingvalues:

- 1 kg cattle dung: 40 litres biogas
- 1 kg buffalo dung: 30 litres biogas
- 1 kg pig dung: 60 litres biogas
- 1 kg chicken droppings: 70 litres biogas

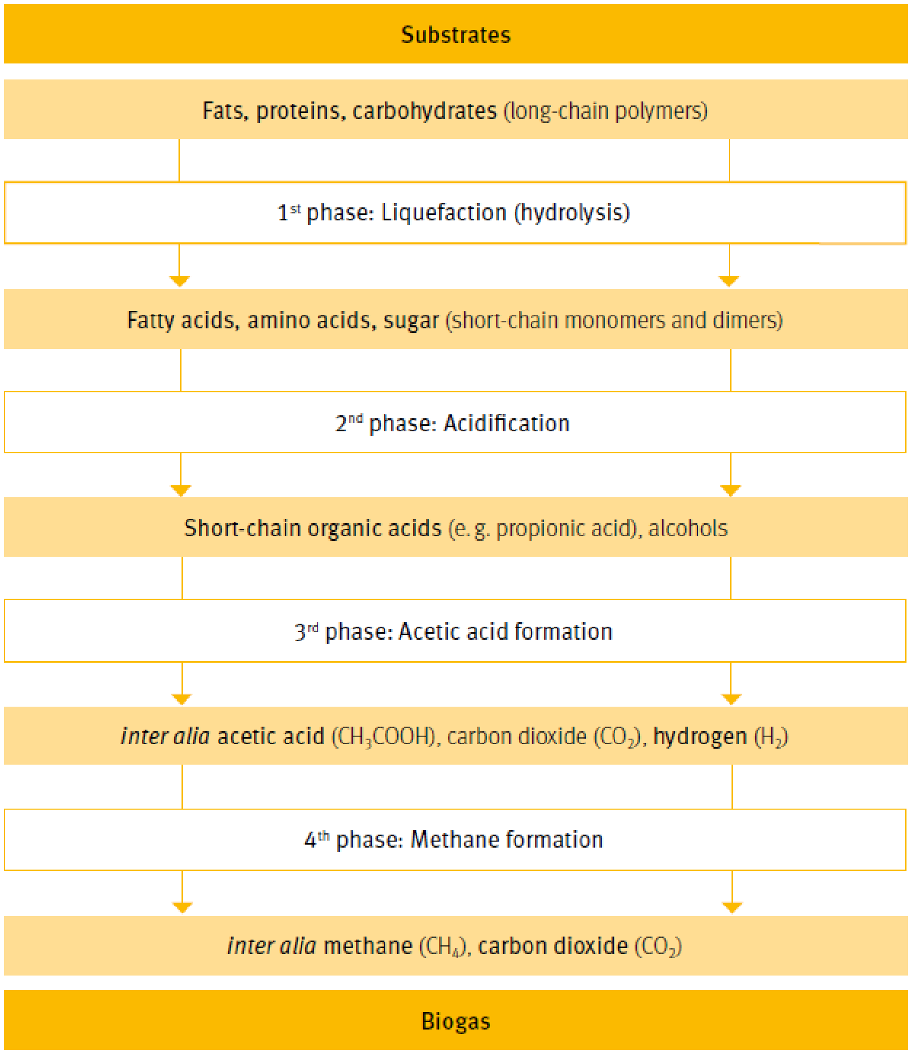


Fig 7. Different Phases of Biogas Production

Electricity Production from Biogas

Theoretically, biogas can be converted directly into electricity by using a fuel cell. However, this process requires very clean gas and expensive fuel cells. The conversion of biogas to electric power by a generator set is much more practical. In contrast to natural gas, biogas is characterized by a high knock resistance and hence can be used in combustion motors with high compression rates. In most cases, biogas is used as fuel for combustion engines which convert it to mechanical energy, powering an electric generator to produce electricity.

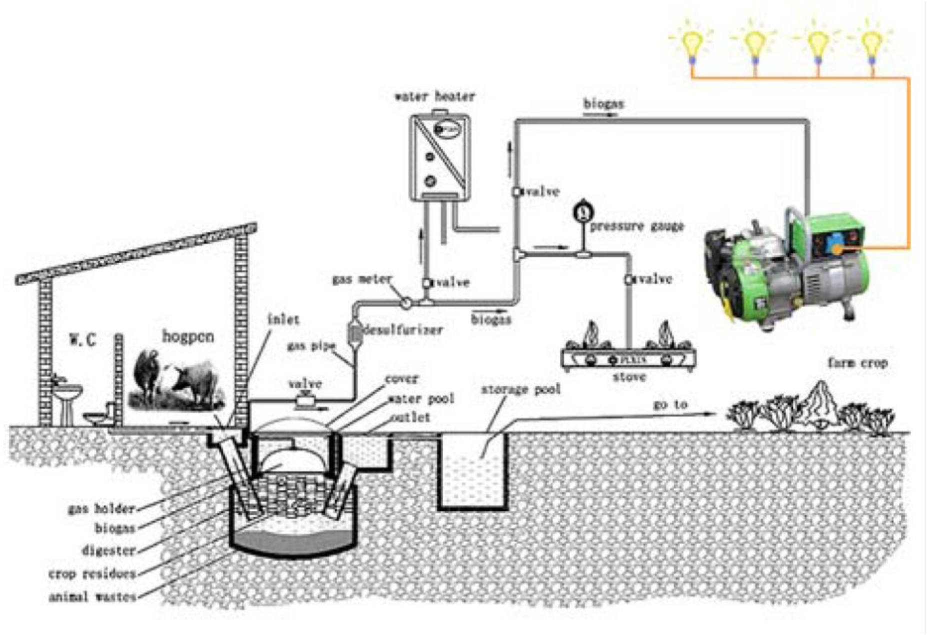


Fig 8. Production of Electricity from Biogas

10.9 Microbial Fuel Cells

A Microbial Fuel Cell (MFC) is a bioreactor that converts chemical energy in the chemical bonds in organic compounds toelectrical energy through catalytic reactions of microorganisms under anaerobic conditions.Like other types of fuel cells, a biological fuel cell consists of an anode, a cathode and a membrane that conducts ions. In the anode compartment, fuel is oxidized by microorganisms and the result is protons and electrons. In the cathode compartment, ions are consumed and the by-product is water. In MFCs, there is the redox reaction between the carbohydrate substrate such as glucose and methanol and the catalysts which is a microorganism or enzyme.The main difference between a standard fuel cell and a BFC is the catalyst is a microorganism or enzyme. Therefore, noble metals are not needed for the catalyst in BFCs. The fuel cell operates in a liquid media in a near neutral environment and at a low temperature. The potential applications for biological fuel cells are:

a) Low power energy sources
b) Sensors based upon direct electrode interactions
c) Electrochemical synthesis of chemicals

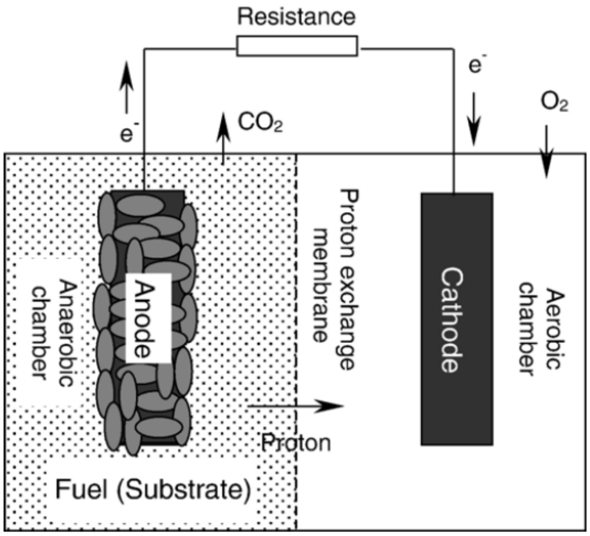


Fig 9. A MFC for Producing Electricity. The anodic and cathodic chambers partitioned by a Proton Exchange Membrane (PEM).

Microbes in the anodic chamber of a MFCoxidize added substrates and generate electrons andprotons in the process. Carbon dioxide is produced asan oxidation product. However, there is no net carbonemission because the carbon dioxide in the renewablebiomass originally comes from the atmosphere in thephotosynthesis process. Unlike in a direct combustionprocess, the electrons are absorbed by the anode andare transported to the cathode through an externalcircuit. After crossing a PEM or a salt bridge, theprotons enter the cathodic chamber where theycombine with oxygen to form water. Microbes inthe anodic chamber extract electrons and protons inthe dissimilative process of oxidizing organic substrates. Electric currentgeneration is made possible by keeping microbeseparated from oxygen or any other end terminalacceptor other than the anode and this requires ananaerobic anodic chamber.

Typical electrode reactions are shown below usingacetate as an example substrate.

$$\text{Anodic reaction : } CH_3COO^- + 2H_2O \xrightarrow{\text{microbes}} 2CO_2 + 7H^+ + 8e^-$$

$$\text{Cathodic reaction : } O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

Two-Compartment MFC System

Two-Compartment MFCs are typically run in batchmode often with a chemically defined medium such asglucose or acetate solution to generate energy. They are currently used only in laboratories. A typical

two compartment MFC has an anodic chamber and a cathodic chamber connected by a PEM, or sometimes a salt bridge to allow protons to move across to the cathode while blocking the diffusion of oxygen into the anode. The compartments can take various practical shapes. They can be useful in powering autonomous sensors for long-term operations in less accessible regions.

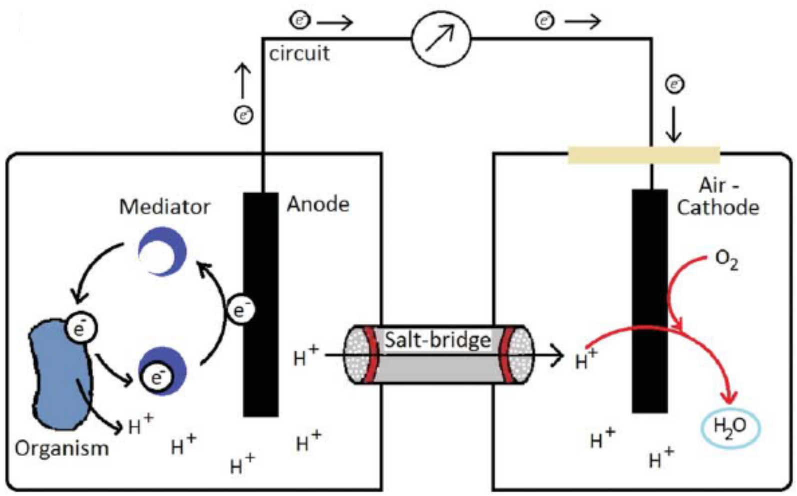


Fig 10 A Two Compartment Microbial Fuel Cell

Single-Compartment MFC systems

Due to their complex designs, two-compartment MFCs are difficult to scale-up even though they can be operated in either batch or continuous mode. One compartment MFCs offer simpler designs and cost savings. They typically possess only an anodic chamber without the requirement of aeration in a cathodic chamber. A one compartment MFC consisting of an anode in a rectangular anode chamber coupled with a porous air-cathode is exposed directly to the external atmosphere. Protons are transferred from the anolyte solution to the porous air-cathode. The anode is made of carbon paper without wet proofing. The cathode is either a carbon electrode/PEM assembly fabricated by bonding the PEM directly onto a flexible carbon-cloth electrode or a standalone rigid carbon paper without PEM. In the absence of a cathodic chamber, catholyte is supplied to the cathode by dripping an electrolyte over the outer woven graphite mat to keep it from drying up.

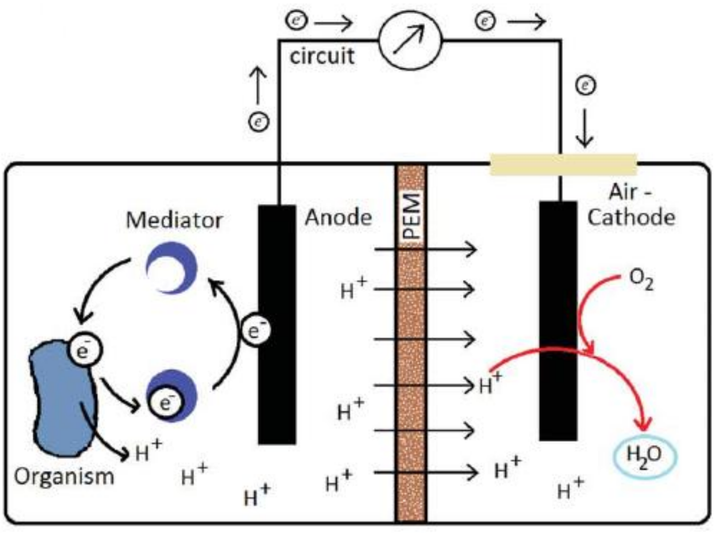


Fig 11 A Single Compartment Microbial Fuel Cell

Hydrogen Production

In fermentation-based systems, microorganisms such as bacteria break down organic matter to produce hydrogen. The organic matter can be refined sugars, raw biomass sources such as corn and even wastewater. Because no light is required, these methods are sometimes called “dark fermentation” methods.

In direct hydrogen fermentation, the microbes produce the hydrogen themselves. These microbes can break down complex molecules through many different pathways and the byproducts of some of the pathways can be combined by enzymes to produce hydrogen. Microbial Electrolysis Cells (MECs) are devices that harness the energy and protons produced by microbes breaking down organic matter combined with an additional small electric current to produce hydrogen.

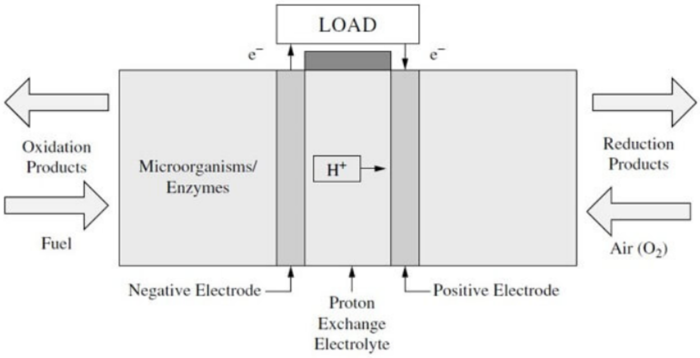


Fig 12 A Schematic Diagram of H_2 Generation from Microbial Fuel Cells

Microbes	Substrate
Actinobacillus succinogenes	Glucose
Aeromonas hydrophila	Acetate
Alcaligenes faecalis, Enterococcus gallinarum, Pseudomonas aeruginosa	Glucose
Clostridium beijerinckii	Starch, glucose, lactate, molasses
Clostridium butyricum	Starch, glucose, lactate, molasses
Desulfovibrio desulfuricans	Sucrose
Erwinia dissolven	Glucose
Escherichia coli	Glucose sucrose
Geobacter metallireducens	Acetate
Geobacter sulfurreducens	Acetate
Gluconobacter oxydans	Glucose
Klebsiella pneumoniae	Glucose
Lactobacillus plantarum	Glucose
Proteus mirabilis	Glucose
Pseudomonas aeruginosa	Glucose
Rhodoferax ferrireducens	Glucose, xylose sucrose, maltose
Shewanella oneidensis	Lactate
Shewanella putrefaciens	Lactate, pyruvate, acetate, glucose
Streptococcus lactis	Glucose

Table 5 Microorganisms used in Microbial Fuel Cells

10.10 Biodiesel

Biodiesel is an alternative fuel similar to conventional or 'fossil' diesel. Biodiesel can be produced from straight vegetable oil, animal oil/fats, tallow and waste cooking oil. The process used to convert these oils to biodiesel is called transesterification. The largest possible source of suitable oil comes from oil crops such as rapeseed, palm or soybean. Though oil straight from the agricultural industry represents the greatest potential source it is not being produced commercially simply because the raw oil is too expensive. Waste vegetable oil can often be sourced for free or sourced already treated for a small price.

Biodiesel Production

Biodiesel is produced using base catalyzed transesterification and it is economical requiring only low temperatures and pressures and producing a 98% conversion yield. The Transesterification process is the reaction of a triglyceride (fat/oil) with an alcohol to form esters and

glycerol. A triglyceride has a glycerine molecule as its base with three long chain fatty acids attached. The characteristics of the fat are determined by the nature of the fatty acids attached to the glycerin. The nature of the fatty acids can in turn affect the characteristics of the biodiesel. During the esterification process, the triglyceride reacts with alcohol in the presence of a catalyst, usually a strong alkaline like sodium hydroxide. The alcohol reacts with the fatty acids to form the mono-alkyl ester or biodiesel and crude glycerol. In most production methods, methanol or ethanol is the alcohol used (methanol produces methyl esters, ethanol produces ethyl esters) and is base catalyzed by either potassium or sodium hydroxide. Potassium hydroxide has been found to be more suitable for the ethyl ester biodiesel production either base can be used for the methyl ester.

The reaction between the fat or oil and the alcohol is a reversible reaction and so the alcohol must be added in excess to drive the reaction towards the right and ensure complete conversion. The products of the reaction are the biodiesel itself and glycerol.

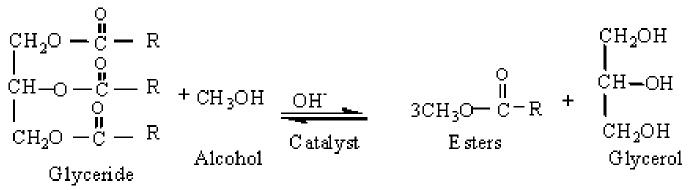


Fig 13 Chemical Process for the Production of Methylester Biodiesel

A successful transesterification reaction is signified by the separation of the ester and glycerol layers after the reaction time. The heavier, co-product, glycerol settles out and may be sold as it is or it may be purified for use in other industries, e.g. the pharmaceutical, cosmetics etc.

Benefits of Biodiesel

Biodiesel has many environmentally beneficial properties. The main benefit of biodiesel is that it can be described as ‘carbon neutral’. This means that the fuel produces no net output of carbon in the form of carbon dioxide (CO_2). This effect occurs because when the oil crop grows it absorbs the same amount of CO_2 as is released when the fuel is combusted. Biodiesel is rapidly biodegradable and completely non-toxic, meaning spillages represent far less of a risk than fossil diesel spillages. Biodiesel has a higher flash point than fossil diesel and so is safer in the event of a crash.

10.11 CHECK YOUR PROGRESS QUESTIONS

1. 1 Define Bio-composting

2. Name the different categories of solid wastes
3. What is aerobic and anaerobic composting? Which of the two is more efficient and faster to produce humus?
4. What are the different microorganisms involved in the process of composting?
5. Of all the techniques available for composting, which are the cheapest and costliest methods?
6. What are the various uses and applications of Bio-compost?
7. What is the prime fuel gas that is produced by Biogas generation?
8. What are the different microorganisms used in Microbial Fuel Cells?
9. Explain ‘transesterification’ reaction in the production of Biodiesel.

10.12 ANSWERS TO CHECK YOUR PROGRESS

1. Bio-composting is a process which converts the organic wastes into humus with the help of mesophilic and thermophilic organisms. It is an economical method to recycle organic wastes with the help of biological agents and an effective method for recovery of nutrients trapped in the organic waste materials. The process of composting seeks to connect the natural forces of decomposition and conversion of organic wastes into organic fertilizers.

2. a) Biodegradable Wastes, b) Recyclable Materials c) Inert Wastes d) Composite Wastes e) Domestic Hazardous Wastes and Toxic wastes.

3. Aerobic and anaerobic composting is the process of composting in the presence and absence of oxygen respectively. Anaerobic decomposition is faster and more effective as it takes place at higher temperature with thermophilic microbes and the temperature as a result of exothermic process helps in the easier breakdown of nutrients.

4. Refer **Table 2**

5. Windrow composting, Aerated Static Piles and In-vessel composting are the three widely used composting technologies. Of these, Windrow composting is the cheapest as it is a passive process that relies on natural process of decomposition, whereas, In-vessel composting is the costliest as it requires specialized equipment to process and handle composting material.

6. Refer Sections 1.7 and 1.8

7. Methane is the prime fuel gas that is produced as a result of Biogas generation. Burning of methane results in the formation of Carbon-di-oxide and Water.
8. Refer Table 5

The Transesterification process is the reaction of a triglyceride (fat/oil) with an alcohol to form esters and glycerol.During the esterification process, the triglyceride reacts with alcohol in the presence of a catalyst, usually a strong alkaline like sodium hydroxide. The alcohol reacts with the fatty acids to form the mono-alkyl ester or biodiesel and crude glycerol. In most production methods, methanol or ethanol is the alcohol used (methanol produces methyl esters, ethanol produces ethyl esters) and is base catalyzed by either potassium or sodium hydroxide

10.13 SUMMARY

Composting is a biological process which converts heterogeneous organic wastes into humus like substances by mixed microbial population under controlled optimum conditions of moisture, temperature and aeration. The composting process starts with the decomposition of easily degradable materials into stabilized material in the presence of oxygen. This leads to the release of heat and subsequent rise in temperature of the compostable material.

Anaerobic composting is a long process and takes place at low temperature and the decomposition of biological material is not efficient resulting in loss of nutrients. Aerobic composting is characterized by rapid decomposition at high temperatures leading to better conversion of nutrients and reduction in foul smelling gases.

Biogas can be produced by anaerobic digestion with anaerobic organisms which digest material inside a closed system or fermentation of biodegradable materials. Biogas is primarily methane and carbon dioxide and may have small amounts of hydrogen sulphide, moisture and siloxanes. The gases methane, hydrogen and carbon monoxide can be combusted or oxidized with oxygen. This energy release allows biogas to be used as a fuel; it can be used for any heating purpose such as cooking. It can also be used in a gas engine to convert the energy in the gas into electricity and heat.

A Microbial Fuel Cell (MFC) is a bioreactor that converts chemical energy in the chemical bonds in organic compounds to electrical energy through catalytic reactions of microorganisms under anaerobic conditions. Like other types of fuel cells, a biological fuel cell consists of an anode, a cathode and a membrane that conducts ions. In the anode compartment, fuel is oxidized by microorganisms and the result is protons and electrons.

Biodiesel is an alternative fuel similar to conventional or ‘fossil’ diesel. Biodiesel can be produced from straight vegetable oil, animal oil/fats, tallow and waste cooking oil. The process used to convert these oils to Biodiesel is called transesterification. The largest possible source of suitable oil comes from oil crops such as rapeseed, palm or soybean.

10.14 KEYWORDS

Bio-composting, Biodiesel, Biogas, Microbial Fuel Cells, Solid Wastes.

10.15 FURTHER READINGS

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UNIT-XI BIOSENSORS

Structure

11.1 INTRODUCTION

The first biosensor was invented in the year 1950 by American biochemist "L.L Clark". This biosensor is used to gauge oxygen in the blood and the electrode used in this sensor is named as Clark electrode or oxygen electrode. Later a gel with glucose oxidize enzyme was layered on the oxygen-electrode to compute blood sugar. Correspondingly, enzyme urease was utilized with an electrode that was invented particularly for NH_4^{++} ions for calculating urea in fluids of the body such as urine and blood. There are three generations of biosensors available in the market. In the first type of biosensor, the reaction of the product disperses to the sensor and causes the electrical reaction. In the second type, the sensor involves in particular mediators between the sensor and the response in order to produce a better response. In the third type, the response itself causes the reaction and no mediator is straightly involved. This article gives an overview of a biosensor**,** working of biosensors, different types and its applications.

11.2 OBJECTIVES

The unit gives clear information to the reader on biosensors and its applications.

11.3 ADVANTAGES OF USING MICROORGANISMS AS BIOSENSING ELEMENTS

Biosensors can be defined as analytical devices which include a combination of biological detecting elements like sensor system and a

transducer. When we compare with any other presently existing diagnostic device, these sensors are advanced in the conditions of selectivity as well as sensitivity. The applications of these biosensors mainly include checking ecological pollution control, in agriculture field as well as food industries. The main features of biosensors are stability, cost, sensitivity and reproducibility.

Animal cells are also in use in a bioreactor. To fulfil the process several perfusion systems have been developed that retain the cells in the bioreactor at the time of replacement of conditioned medium with fresh medium. This results in increase in cell density and in turn cell productivity. For commercial production of products, a large scale cell culture system and scaling up of process are required.

Some of the important products which are produced from animal cell cultures are: (i) enzymes (asperagenase, collagenase, urokinase, pepsin, hyaluronidase, rennin, trypsin, tyrosine hydroxylase), (ii) hormones (leutinizing hormone, follicle stimulating hormone, chorionic hormone and erythropoietin), (iii) vaccines (iv) monoclonal antibodies and (v) interferons.

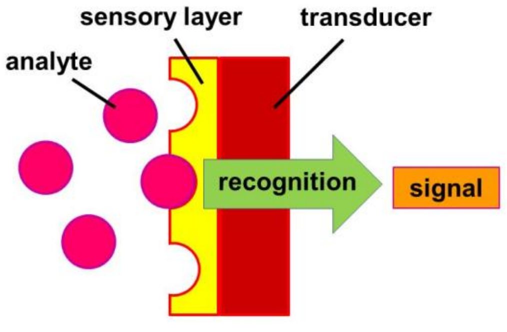


Fig. 1 Mechanism of a Biosensor

11.3.1 Main Components of a Biosensor

The block diagram of the biosensor includes three segments namely, sensor, transducer, and associated electrons. In the first segment, the sensor is a responsive biological part, the second segment is the detector part that changes the resulting signal from the contact of the analyte and for the results it displays in an accessible way. The final section comprises of an amplifier which is known as signal conditioning circuit, a display unit as well as the processor.

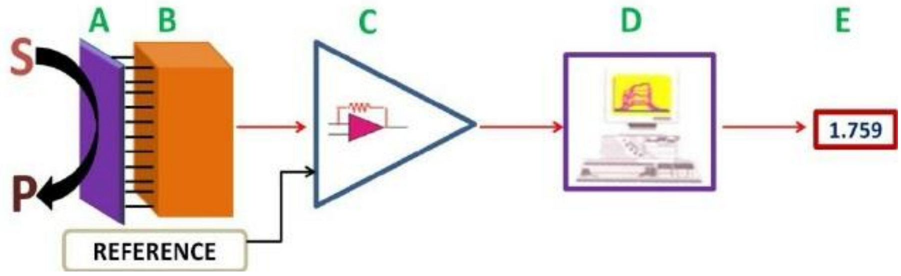


Fig. 2 Components of a Biosensor

11.3.2 Working Principle of Biosensors

Usually, a specific enzyme or preferred biological material is deactivated by some of the usual methods and the deactivated biological material is in near contact to the transducer. The analyte connects to the biological object to shape a clear analyte which in turn gives the electronic reaction that can be calculated. In some examples, the analyte is changed to a device which may be connected to the discharge of gas, heat, electron ions or hydrogen ions. In this, the transducer can alter the device linked converts into electrical signals which can be changed and calculated.

Working of Biosensors

The electrical signal of the transducer is frequently low and overlay upon a fairly high baseline. Generally, the signal processing includes deducting a position baseline signal obtained from a related transducer without any biocatalyst covering. The comparatively slow character of the biosensor reaction significantly eases the electrical noise filtration issue. In this stage, the direct output will be an analog signal however it is altered into digital form and accepted to a microprocessor phase where the information is progressed, influenced to preferred units and o/p to a data store.

11.3.3 Microorganisms, Biosensors and its Advantages

Biomolecules such as enzymes, antibodies, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Among these, microorganisms offer advantages of ability to detect a wide range of chemical substances, amenability to genetic modification and broad operating pH and temperature range making them ideal as biological sensing materials. Microorganisms have been integrated with a variety of transducers such as amperometric, potentiometric, calorimetric, conductimetric, colorimetric, luminescence and fluorescence to

construct biosensor devices. Several review papers and book chapters addressing microbial biosensor development have been published.

Advantages of using Microorganisms as Biosensing Elements

Enzymes are the most widely used biological sensing element in the fabrication of biosensors. Although purified enzymes have very high specificity for their substrates or inhibitors, their application in biosensor construction may be limited by the tedious, time-consuming and costly enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/coenzyme. Microorganisms provide an ideal alternative to these bottle-necks. Many enzymes and co-factors that co-exist in the cells give the cells the ability to consume and hence detect large number of chemicals; however, this can compromise the selectivity. They can be easily manipulated and adapted to consume and degrade new substrate under certain cultivating condition. Additionally, the progress in molecular biology/recombinant DNA technologies has opened endless possibilities of tailoring the microorganisms to improve the activity of an existing enzyme or express foreign enzyme/protein in host cell. All of these make microbes excellent biosensing elements.

11.4 IMMOBILIZATION OF MICROORGANISMS

The basis of a microbial biosensor is the close contact between microorganisms and the transducer. Thus, fabrication of a microbial biosensor requires immobilization on transducers with a close proximity. Since microbial biosensor response, operational stability and long-term use are to some extent a function of the immobilization strategy used, immobilization technology plays a very important role and the choice of immobilization technique is critical. Microorganisms can be immobilized on transducer or support matrices by chemical or physical methods.

Chemical Methods

Chemical methods of microbe immobilization include covalent binding and cross-linking. Covalent binding methods rely on the formation of a stable covalent bond between functional groups of the microorganism’s cell wall components such as amine, carboxylic or sulphydryl and the transducer such as amine, carboxylic, epoxy or tosyl. To achieve this goal, whole cells are exposed to harmful chemicals and harsh reaction condition which may damage the cell membrane and decrease the biological activity. The way to overcome this drawback is

still a challenge for immobilization through covalent binding. To our knowledge, this method has therefore not been successful for immobilization of viable microbial cells. Cross-linking involves bridging between functional groups on the outer membrane of the cells by multifunctional reagents such as glutaraldehyde and cyanuric chloride to form a network. Because of the speed and simplicity, the method has found wide acceptance for immobilization of microorganisms. The cells may be cross-linked directly onto the transducer surface or on a removable support membrane which can then be placed on the transducer. The ability to replace the membrane with the immobilized cells is an advantage of the latter approach. While cross-linking has advantages over covalent binding, the cell viability and/or the cell membrane biomolecules can be affected by the cross-linking agents. Thus cross-linking is suitable in constructing microbial biosensors where cell viability is not important and only the intracellular enzymes are involved in the detection.

Physical Methods

Adsorption and entrapment are the two widely used physical methods for microbial immobilization. Because these methods do not involve covalent bond formation with microbes and provide relatively small perturbation of microorganism native structure and function, these methods are preferred when viable cells are required. Physical adsorption is the simplest method for microbe immobilization. Typically, a microbial suspension is incubated with the electrode or an immobilization matrix such as alumina and glass bead followed by rinsing with buffer to remove unadsorbed cells. The microbes are immobilized due to adsorptive interactions such as ionic, polar or hydrogen bonding and hydrophobic interaction. However, immobilization using adsorption alone generally leads to poor long-term stability because of desorption of microbes. The immobilization of microorganisms by entrapment can be achieved by the either retention of the cells in close proximity of the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels such as alginate, carrageenan, agarose, chitosan, collagen, polyacrylamide, polyvinylachohol, polyethylene glycol, polyurethane, etc. A major disadvantage of entrapment immobilization is the additional diffusion resistance offered by the entrapment material which will result in lower sensitivity and detection limit. Microbial biosensor can be classified based on the transducers into electrochemical, optical and others.

11.4 ELECTROCHEMICAL MICROBIAL BIOSENSOR

There are three types of electrochemical microbial biosensors: amperometric, potentiometric and conductometric

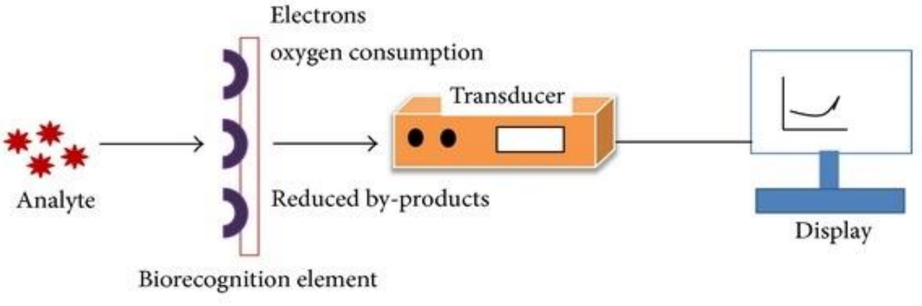


Figure3: Electrochemical Microbial Biosensor

Amperometric Microbial Biosensor

Amperometric microbial biosensor operates at fixed potential with respect to a reference electrode and involves the detection of the current generated by the oxidation or reduction of species at the surface of the electrode. Amperometric microbial biosensors have been widely developed for the determination of biochemical oxygen demand (BOD) for the measurement of biodegradable organic pollutants in aqueous samples. The conventional standard method for the determination of BOD measures the microorganism's oxygen consumption/respiration over a period of 5 days and is reported as BOD5. While BOD5 is a good indicator of the concentration of organic pollutants in water, it is extremely slow and hence not suitable for process control. To address this limitation, several BOD biosensors based on amperometric oxygen electrode transducer modified with microorganisms degrading/metabolizing organic pollutants have been reported. The microbial strains used as biological sensing element include *Torulopsis candida*, *Trichosporon cutaneum*, *Pseudomonas putida*, *Klebsiella oxytoca* AS1, *Bacillus subtilis*, *Arxula adeninivorans* LS3, *Serratia marcescens* LSY4, *Pseudomonas* sp., *P. fluorescens*, *P. putida* SG10, *Thermophilic bacteria*, *Hansennula anomala* and yeast. Because any given strain provides a narrow substrate spectrum, single strain-BOD-biosensor has limitations in analyzing complex samples. This bottleneck can be alleviated by employing a mixture of two or more microorganisms to broaden the substrate and hence analyte spectrum with a stable performance. As the most extensively investigated microbial biosensor, the first commercial BOD biosensor was produced by Nisshin Denki

(Electric) in 1983. After which several more BOD biosensors have been commercialized by DKK Corporation, Japan; Autoteam FmbH, Germany; Prufgeratewerk Medingen GmbH, Germany; Dr. Lange GmbH, Germany; STIP Isco GmbH, Germany; Kelma, Belgium; LAR Analytik and Umweltmesstechnik GmbH, Germany; Bioscience, Inc., USA; USFilter, USA. While most of the research and development in BOD biosensors has focused in identifying different microorganisms that can determine BOD of a specific waste, research efforts have also been directed at improving the amperometric transducer itself. For example, a miniaturized oxygen electrode based on thick-film screen-printing was recently developed to replace the bulky Clark dissolved oxygen electrode transducer. The widely used thick-film screen-printing technique was used to print the platinum-working electrode, Ag/AgCl reference electrode and platinum auxiliary electrode of the amperometric oxygen electrode on an inert substrate. The oxygen electrode was then modified with *A. adeninivorans* LS3 by entrapment in Poly Carbamoyl Sulfonate (PCS) gel and successfully applied for rapid (~100 s) and stable (upto 2 months) BOD determination. Similarly, to extend the dynamic range of the BOD sensor which in the case of dissolved oxygen electrode is limited by the solubility of oxygen in the sample, a ferricyanide mediated microbial biosensor using a novel yeast strain for BOD measurement was developed. Recently, an amperometric transducer array featuring four individually addressable platinum electrodes was constructed and modified with two microbial strains with different substrate spectra for the measurement of BOD and polycyclic aromatic hydrocarbons (PAH) simultaneously.

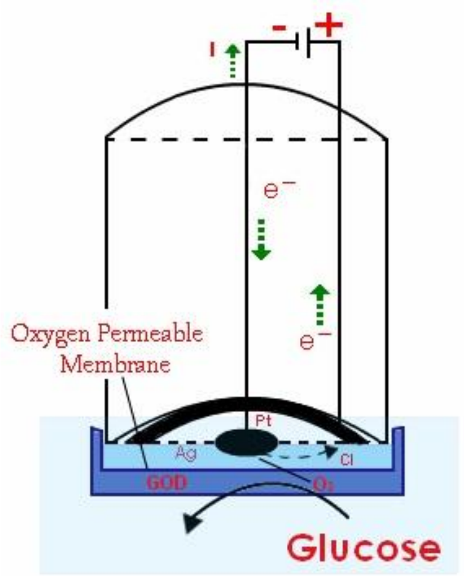


Fig. 4 Amperometric Microbial Biosensor

Besides BOD biosensor, amperometric microbial biosensors have also been applied for measurement of several other chemicals. Because of its importance in fermentation industry and clinical

toxicology, microbial biosensor for ethanol has garnered the second most research attention after BOD. Different microorganisms metabolizing ethanol such as *Trichosporon brassicae*, *Acetobacter aceti*, *Candida vini*, *Gluconobacter suboxydans*, *C. tropicalis*, *Aspergillus niger*, *Saccharomyces ellipsoideus*, *G. oxydans* and *Pichia methanolica* have been immobilized on oxygen electrode to fabricate ethanol biosensors. While these biosensors possess good sensitivity and stability but have poor selectivity. Thus, there is a great interest to develop selective microbial ethanol biosensor. An improved selectivity for ethanol determination in the presence of glucose was achieved by replacing oxygen with ferricyanide as the electron acceptor mediator for *G. oxydans* immobilized on a glassy carbon electrode by cellulose acetate membrane which also restricted the availability of glucose to the cells by size exclusion. Sugars are important ingredients of different media and for their determination sensors are therefore highly desired. Microbial biosensors for sugars have ranged from the simple modification of Clark and microfabricated oxygen electrode with *S. cerevisiae* and *E. coli* K12 mutants respectively to modification of graphite electrode with *G. oxydans* in conjunction with hexacyanoferrate (III) as a mediator. Phenol and substituted phenols have received considerable attention in waste analysis program due to their high toxicity to mammals, humans and plants. A variety of amperometric microbial biosensors have been reported for these EPA priority chemicals. p-Nitrophenol (PNP) degrading bacterial *Arthrobacter* JS 443 and *Moraxella sp.* isolated from PNP contaminated sites in the U.S. have been immobilized on oxygen and carbon paste electrodes using polycarbonate membrane and Nafion respectively and by directly mixing in the carbon paste to fabricate biosensor for PNP.

Other microbial biosensors for phenols include *Rhodococcus erthropolis* modified Clark oxygen electrode for 2,4-dinitrophenol (2,4-DNP) and *P. putida* DSM 50026, a well-known phenol degrading microorganisms, modified thick-film and screen printed graphite electrodes for phenols. Neurotoxic organophosphate (OP) compounds have found wide applications as pesticides and insecticides in agriculture and as chemical warfare agents in military practice. Amperometric biosensors based on genetically engineered *Moraxella* sp. and *P. putida* with surface-expressed organophosphorus hydrolase (OPH) have been developed for sensitive, selective and cost-effective detection of OPs. These biosensors rely on the amperometric detection of PNP generated from hydrolysis of OP compounds by surface-displayed OPH or oxygen consumed and electrochemically active intermediates formed during the further mineralization of the PNP by the cells. The inhibition of bacterial respiration and hence the decrease of oxygen consumption rate has been utilized to fabricate cyanide biosensor. Whole-cell biosensors consisting of dissolved oxygen electrode modified with *Nitrosomonas europaea*, *Thiobacillus ferrooxidans*, *Saccharomyces cerevisiae* and *Pseudomonas*

fluorescens were reported for batch and continuous cyanide monitoring. Other amperometric microbial biosensors based on monitoring of cell respiration include biosensor for surfactants representing a widespread group of organic pollutants using surfactant-degrading bacteria, hydrogen peroxide by coupling immobilized living *Acetobacter peroxydans* and for acetic acid using *Fusarium solani*. Over the last two decades, the microbiologically influenced corrosion (MIC) of metallic materials has received great attention. A stable, reproducible and specific microbial biosensor was developed for monitoring MIC of metallic materials in industrial systems based on *Pseudomonas sp*. isolated from corroded metal surface and immobilized on acetylcellulose membrane at oxygen electrode. A linear relationship between the biosensor response and the concentration of sulfuric acid, the most corrosive inorganic acid involved in microbial corrosion was established. The biosensor response time was 5 min and was dependent on many parameters such as pH, temperature, corrosive environment and immobilized cell loading. The same group also used *Acetobacter* sp. to develop amperometric microbial biosensor for monitoring microbiologically influenced corrosion caused by fungal species.

Another application of amperometric microbial biosensors is the detection of heavy metal ions for environmental control. A microbial biosensor to detect Cu^{2+} by an amperometric method has been developed using recombinant *S. cerevisiae* containing plasmids with Cu^{2+}- inducible promoter fused to the lacZ gene. In the presence of Cu^{2+}, the recombinant strains are able to utilize lactose as a carbon source and lead to the oxygen consumption change which can be detected by using oxygen electrode. A novel promoter-based electrochemical biosensor for on-line and in situ monitoring of gene expression in response to cadmium has also been described. A cadmium-responsive promoter from *E. coli* was fused to a promoterless lacZ gene and the β-galactosidase activity was monitored using screen-printed electrode in the presence of cadmium. This whole-cell biosensor could detect nanomolar concentrations of cadmium on-line or in-site within minutes.

Potentiometric Microbial Biosensor

Conventional potentiometric microbial biosensors consist of an ion-selective electrode (pH, ammonium, chloride) or a gas-sensing electrode (pCO_2 and pNH_3) coated with an immobilized microbe layer. Microbe consuming analyte generates a change in potential resulting from ion accumulation or depletion. Potentiometric transducers measure the difference between a working electrode and a reference electrode and the signal is correlated to the concentration of analyte. Due to a logarithmic relationship between the potential generated and analyte concentration, a wide detection range is possible. However, this method requires a very stable reference electrode which may be a limitation of

these transducers. A few examples of biosensors based on potentiometric transducers are elaborated. The simplest potentiometric microbial biosensor is based on the modification ion selective electrode.

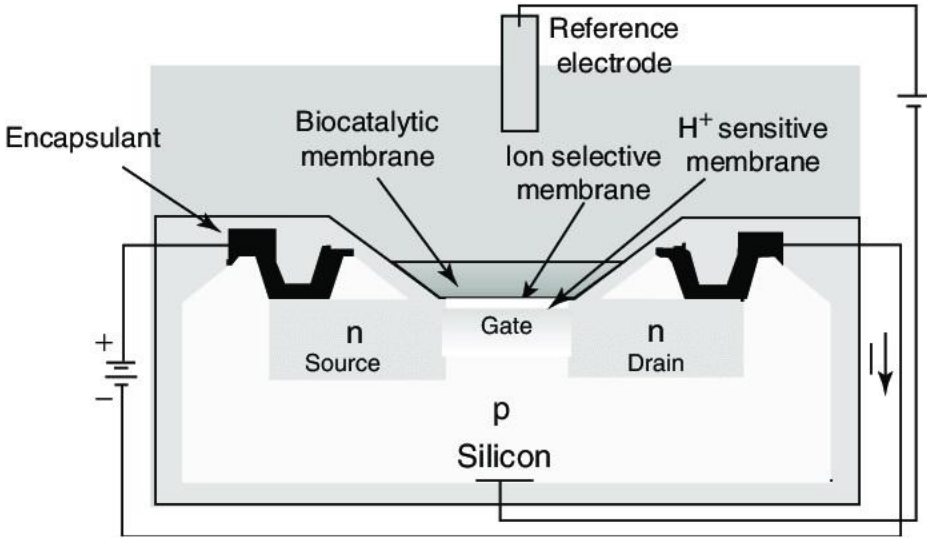


Fig 5: Potentiometric Microbial Biosensor

Several microbial biosensors based on modification of glass pH electrode with genetically engineered *E. coli* expressing organophosphorus hydrolase intracellularly and on the outer surface of cells and wild-type OP degrading bacteria *Flavobacteium sp*. have been reported. The principle of detection is based on the detection of the protons released by OPH catalyzed hydrolysis of OP and correlating to the concentration of OPs. Similarly, recombinant *E. coli* harboring the plasmids encoding for β-lactamase and penicillinase synthesis immobilized on pH electrode using gluten and acetylcellulose membranes entrapment respectively were developed for monitoring penicillin. A new type of solid state silicon-based light addressable potentiometric sensor for monitoring hydrogen ion was integrated to the auxotrophic bacteria *E.coli* WP2 requiring tryptophan for its growth to fabricate a potentiometric microbial assay for tryptophan.

While pH electrodes are the most widely applied ion selective electrode for microbial biosensors, other ion selective electrodes have also been utilized. For example, an ammonium ion selective electrode was coupled with urease-yielding *Bacillus* sp. isolated from soil to develop a disposable microbial biosensor for monitoring the presence of urea in milk. Similarly, a chloride ion selective electrode was modified with TCE degrading bacterium, *Pseudomonas aeruginosa* JI104 for TCE

monitoring in batch and continuous modes in wastewaters. A potentiometric oxygen electrode with immobilized *S. ellipsoideus* was also successfully used to produce a microbial biosensor for the determination of ethanol with an extended response range. Based on the same format, sucrose biosensor based on an immobilized *S. cerevisiae* was also described.

Conduct metric Biosensor

Many microbe-catalyzed reactions involve a change in ionic species. Associated with this change is a net change in the conductivity of the reaction solution. Even though the detection of solution conductance is non-specific, conductance measurements are extremely sensitive. Recently, a single-use conductivity and microbial sensor were developed to investigate the effect of both species and concentration/osmolarity of anions on the metabolic activity of *E. coli*. This hybrid sensing system combines physico-chemical and biological sensing and greatly increases the ease with which comparative data could be assimilated.

Optical Microbial Biosensor

The modulation in optical properties such as UV-Vis absorption, bio and chemiluminescence, reflectance and fluorescence brought by the interaction of the biocatalyst with the target analyte is the basis for optical microbial biosensors. Optical based biosensors offer advantages of compactness, flexibility, resistance to electrical noise and a small probe size.

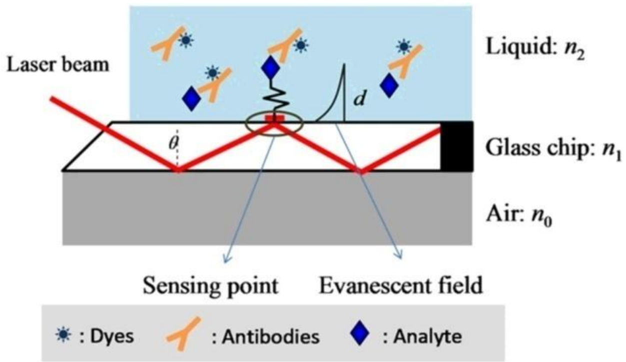


Fig. 6 Optical Microbial Biosensor

Bioluminescence Biosensor

Bioluminescence is associated with the emission of light by living microorganisms and it plays a very important role in real time process monitoring. The bacterial luminescence lux gene has been widely applied as a reporter either in an inducible or constitutive

manner. In the inducible manner, the reporter lux gene is fused to a promoter regulated by the concentration of a compound of interest. As a result, the concentration of the compound can be quantitatively analyzed by detecting the bioluminescence intensity. In the constitutive manner, the reporter gene is fused to promoters that are continuously expressed as long as the organism is alive and metabolically active. This kind of reporter is good for evaluating the total toxicity of contaminant. Both types of reporters have been shown to be useful for biosensor development. Heavy metal-mediated toxicity in the environment is dependent on bioavailable metal concentrations. Bioluminescent microbial biosensors have been extensively investigated to monitor bioavailable metal. *Ralstonia eutropha* AE2515 was constructed by transcriptionally fusing cnrYXH regulatory genes to the bioluminescent *lux*CDABE report system to fabricate a whole cell biosensor for the detection of bioavailable concentration of Ni^{2+} and Co^{2+} in soil. Several optical biosensors consisting of bacteria that contain gene fusion between the regulatory region of the mer operon (*mer*R) and *lux*CDABE have been developed to quantitatively respond to Hg^{2+}. The mer promoter is activated when Hg^{2+} binds to *mer*R which results in the transcription of the lux reporter gene and subsequently the light emission. Bioavailable copper in soil is also monitored by using engineered *P. fluorescens* through mutagenesis of *P. fluorescens* containing copper-induced gene and Tn5::*lux*AB promoter probe transposon. In order to monitor nutrients in an aquatic ecosystem, a biosensor for monitoring phosphorus bioavailablity to Cyanobacteria (*Synechococcus* PCC 7942) was developed. The reporter strain, *Synechococcus* harbors the gene coding the reporter protein luciferase under the control of an inducible alkaline phosphatase promoter which can be induced under phosphorous limitation and shows improvement to the conventional phosphorus detection methods. Bioluminescent microbial biosensors using the inducible reporter gene have also been developed for the measurement of bioavailable naphthalene, tributyltin and halogenated organic acids.

The environmental problems caused by industrial and agricultural pollution have increased the demand for the development of pollutant and toxicity detection methods. The fusion of reporter genes to promoters that are induced when cell are stressed by toxic chemicals are one promising approach that has been used to fabricate biosensor for such application. Recombinant *E. coli* bearing fabA'::lux fusion and plasmid pUCD607 containing the full luxCDABE cassette have been constructed as biosensors for water pollutant detection. The online pollutant and toxicity test using bioluminescence-based biosensors was proved to be sensitive and reliable. Lux-marked R*hizobacterium P. fluorescens* has been developed to evaluate the pollution-induced stress which influences *Rhizobacterium* carbon flow based on the fact that bioluminescence output of biosensor is directly correlated with

metabolic activity and reports on carbon flow in root exudates. Furthermore, lux-marked whole cell biosensors for evaluation of interactive toxicity of chlorophenol and toxicity assessment of a wastewater treatment plant treating phenolics containing waste have been reported respectively. These biosensors respond to test pollutants fast and enable a rapid toxicity test possible.

Genotoxicants is a class of hazards which can cause DNA damage. An optical-fiber bioluminescent microbial sensor to detect the DNA damage hazard-mitomycin C by the induction of a selected promoter and the subsequent production of bioluminescent light through a recombinant lux reporter was reported. Bioluminescence production was shown to be dose dependent. *E. coli* containing plasmid-borne fusion of the recA promoter-operator region to the *Vibrio fischeri* lux genes has also been reported for genotoxicant detection. When the recombinant *E. coli* strains are challenged with DNA damage hazards, they increase their luminescence.

Fluorescence Biosensor

Fluorescence spectroscopy has been widely applied in analytical chemistry. It is a sensitive technique that can detect very low concentrations of analyte because of the instrumental principles involved. At low analyte concentrations, fluorescence emission intensity is directly proportional to the concentration. Fluorescent materials and green fluorescent protein have been extensively used in the construction of fluorescent biosensor.

Green Fluorescence Protein-based Biosensor

As bioluminescent reporter lux gene, gfp gene coding for the green fluorescent protein (GFP) has also been widely applied as reporters and fused to the host gene that allows reporter activity to be examined in the individual cells. Because GFP is very stable and not known to be produced by microorganism indigenous to terrestrial habits, it provides great advantage and flexibility when evaluating the reporter activity. The primary disadvantage of GFP as a reporter protein is the delay between protein production and protein fluorescence.

The GFP-based microbial biosensor has been shown to be useful in assessing heterogeneity of iron bioavailability on plant. In this sensor, ferric iron availability to cells was assessed by quantifying the fluorescence intensity of cells containing a plasmid-borne transcriptional fusion between an ion-regulated promoter and GFP. Recently, Wells *et al.*, (2005) developed an ultrasensitive biosensor for arsenite by using laser-induced fluorescence confocal spectroscopy to

measure arsenite-stimulated enhanced green fluorescent protein synthesis of genetically engineered *E. coli* bioreporter cell which has an inherent single-molecule detection capability. A recombinant soil bacterium, *Sinorhizobium meliloti* has been constructed by fusing the gfp gene to the melA promoter which is induced on exposure to galactose and galactosides. Using this fusion strain, a biosensor was developed to determine the concentration of galactosides. Similarly, gfp reporter gene has also been used to develop biosensors for various applications such as detecting bioavailable toluene and related compounds and N-acyl homoserine lactones in soil measuring water availability in a microbial habitat, monitoring cell populations etc. With the development of DNA recombinant technologies and our understanding to microbes, this type of biosensor will become an increasingly more powerful technique.

O_2-sensitive Fluorescent Material-based Biosensor

Besides green fluorescent protein, other fluorescent materials have also been used in the construction of microbial biosensor. Recently, fiber-optical microbial sensors for determination of BOD were reported. The biosensors consists of either a layer of oxygen-sensitive fluorescent materials that are made up of seawater microorganisms immobilized in polyvinyl alcohol sol-gel matrix and an oxygen fluorescence quenching indicator with linear range of 4-200 mg/l or an immobilized *P. putida* membrane attached to an optical fiber sensor for dissolved oxygen from ASR Co. Ltd. with a detection limit of 0.5 mg/l.

Colorimetric Biosensor

A sensitive biosensor based on color changes in the toxin sensitive colored living cells of fish has been reported. In the presence of toxins produced by microbial pathogens, the cells undergo visible color change and the color changes in a dose-dependent manner. The results suggest that this cell-based biosensor's potential application lies in the detection and identification of virulence activity associated with certain air, food and water-borne bacterial pathogens. A simple fiber-optic based microbial sensor is also reported to detect organophosphates based on the absorbance of PNP formed from the hydrolysis of organophosphates by the genetically engineered *E. coli* expressing organophosphorus hydrolase on the cell surface. This biosensor can be easily extended to other organophosphates such as coumaphos through the monitoring of its hydrolysis product coumarin. A colorimetric whole cell bioassay for the detection of the common environmental pollutants benzene, toluene, ethyl benzene and xylene (BTEX) has been found at underground fuel storage tanks using recombinant *E. coli*

expressing toluene dioxygenase and toluene dihydrodiol dehydrogenase. The bioassay was based on the enzyme catalyzed conversion of the BTEX components to their respective catechols followed by the reaction with hydrogen peroxide in presence of horseradish peroxidase to colrimetric products that can be monitored at 420 nm.

11.6 Other Types of Microbial Biosensors

Besides electrochemical, optical and colorimetric microbial biosensors there are few other types of biosensors reported recently.

Baroxymeter Biosensor

Sensors based on baroxymeter for the detection of pressure change. As a new application, baroxymeter has been developed as a portable wastewater direct toxicity assessment device based on manometric bacterial respirometry. The pressure drop in the headspace of a close vessel due to oxygen uptake by the microorganism in contact with sample will be measured by the respirometry. This microbial pressure sensor will exhibit good reproducibility and comparable responses with other reported methods.

Infrared analyzer Biosensor

Sensors based on infrared analyzer for the detection of the microbial respiration product CO_2. A new method has been reported for monitoring the inhibitory effects in wastewater treatment plants based on the continuous measurement of the microbial respiration product CO_2. Activated sludge microbes are used as the biological elements and their respiratory activity is inhibited by the presence of toxic compounds resulting in a decrease in CO_2 concentration which can be analyzed by using a CO_2 infrared analyzer. Based on the measurement of CO_2 concentration in the off gas produced during degradation of carbon compound by microbial respiration activities, a microbial biosensor has been developed to monitor the extent of organic pollution in wastewater both off-line in a laboratory and online in a wastewater treatment plant.

11.7 CHECK YOUR PROGRESS QUESTIONS

1. Biosensor
2. Immobilization
3. Conductimetric Biosensor
4. Colorimetric Biosensor

11.8 ANSWERS TO CHECK YOUR PROGRESS

1. 1. Cytokines are a large group of proteins, peptides or glycoproteins A device which uses a living organism or biological molecules especially enzymes or antibodies to detect the presence of chemicals
2. The technique used for the physical or chemical fixation of cells, organelles, enzymes or other proteins (e.g. monoclonal antibodies) onto a solid support, into a solid matrix or retained by a membrane in order to increase their stability and make possible their repeated or continued use.
3. Transducers for conductometric biosensors. The conductometric transducer is a miniature of two electrolyte layer adjacent to the electrode surface.
4. A biosensor is an analytical device which converts a biological response into an electrical signal. Most of this current endeavour concerns potentiometric and amperometric biosensor and colorimetric paper enzyme strips.

11.9 SUMMARY

✓ A biosensor is a device that measures biological or chemical reaction by generating signals proportional to the concentration of an analyte in the reaction. Analyte is a substance of interest that needs detection. For instance, glucose is an ‘analyte’ in a biosensor designed to detect glucose.

✓ Distinct advantages that enzyme-based biosensors provide such as high sensitivity and specificity, portability, cost-effectiveness and the possibilities for miniaturization and point of care diagnostic testing make them more and more attractive for research focused on clinical analysis, food safety control or disease monitoring purposes.

✓ Electrochemical biosensors play a critical role in the advancement of commercial point of care systems with detectors for glucose, uric acid and cholesterol currently available as diagnostic devices.

✓ A microbial biosensor is an analytical device that immobilizes microorganisms onto a transducer for the detection of target analytes. With the development of nanotechnology, nonomaterials have been used to achieve better immobilization for developing a more reliable and selective microbial biosensor.

11.10 KEYWORDS

BOD, Cell Bioassay, Green Fluorescent Protein, Lux Gene

11.11 SELF ASSESSMENT QUESTIONS & EXCERCISES

Short Answer Questions

1. Write a brief note on Biosensors.
2. Mention the advantages of using microorganisms as biosensing elements.
3. Differentiate Amperometric and Potentiometric Microbial Biosensor.
4. Comment on Microbial Fuel Cell Type Biosensor.
5. Bioluminescence Biosensor.

Longer Answer Questions

1. In detail explain about Immobilization of microorganisms.
2. Applications of Microbial Biosensor.
3. Comment on Electrochemical Microbial Biosensor with its types.
4. What do you know about Optical Microbial Biosensor?
5. Make a note on Other Types of Microbial Biosensors.

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UNIT-XII
GENETICALLY MODIFIED MICROORGANISMS

Structure

12.1 INTRODUCTION

With the development of recombinant deoxyribonucleic acid (DNA) technology, the metabolic potentials of microorganisms are being explored and harnessed in a variety of new ways. Today, genetically modified microorganisms (GMMs) have found applications in human health, agriculture and bioremediation and in industries such as food, paper and textiles. Genetic engineering offers the advantages over traditional methods of increasing molecular diversity and improving chemical selectivity. In addition, genetic engineering offers sufficient supplies of desired products, cheaper product production and safe handling of dangerous agents. This chapter delineates several molecular tools and strategies to engineer microorganisms; the advantages and limitations of the methods are addressed. The final part of this chapter reviews and evaluates several applications of GMMs currently employed in commercial ventures.

12.2 OBJECTIVES

This Unit provides the reader with information pertaining to genetically modified microorganisms and molecular tools for genetic engineering of microorganisms.

12.3 MOLECULAR TOOLS FOR GENETIC ENGINEERING OF MICROORGANISM

A number of molecular tools are needed to manipulate microorganisms for the expression of desired traits. These include:

(1) Gene transfer methods to deliver the selected genes into desired hosts.
(2) Cloning vectors.
(3) Promoters to control the expression of the desired genes.
(4) Selectable marker genes to identify recombinant microorganisms.

12.3.1 Gene Transfer Methods

The most frequently used method is transformation. In this process, uptake of plasmid DNA by recipient microorganisms is accomplished when they are in a physiological stage of competence which usually occurs at a specific growth stage. However, DNA uptake based on naturally occurring competence is usually inefficient. Competence can be induced by treating bacterial cells with chemicals to facilitate DNA uptake. For *Escherichia coli*, an organism used commonly as a cloning host and a "bioreactor" for the commercial production of numerous therapeutic proteins, the uptake of plasmid DNA is achieved when cells are first treated with calcium chloride or rubidium chloride.

For many microorganisms such as the antibiotic producing Streptomyces, transformation of plasmid DNA is a more complicated process. For these organisms, transformation involves preparation of protoplasts using lysozyme to remove most of the cell wall. Protoplasts are mixed with plasmid DNA in the presence of polyethylene glycol to promote the uptake of DNA. Growth medium, growth phase, ionic composition of transformation buffers and polyethylene glycol molecular weight, concentration and treatment time are variables that must be studied to identify the optimum conditions for protoplast formation and regeneration. Electroporation is an alternative method to transform DNA into microorganisms. This method is originally used to transform eukaryotic cells and relies on brief high-voltage pulses to make recipient cells electrocompetent. Transient pores are formed in the cell membrane as a result of an electroshock, thereby allowing DNA uptake. Growth phase, cell density, growth medium and electroporation parameters must be optimized to achieve desirable efficiency. The main advantage of this method is that it bypasses the need to develop conditions for protoplast formation and regeneration of cell wall. Electroporation is often used when the efficiency of protoplast transformation is insufficient or ineffective. Several reports have

documented the application of this method to industrially important *Streptomyces*, *Corynebacterium* and *Bacillus*. Electroporation is also the primary method of choice for transferring DNA into lactic acid bacteria. In addition to using purified DNA for electroporation procedures, methods have been developed to transfer DNA directly from DNA-harboring cells into a recipient without DNA isolation.

Conjugation is another method used to introduce plasmid DNA into microorganisms. This method involves a donor strain that contains both the gene of interest and the origin of transfer (oriT) on a plasmid and the genes encoding transfer functions on the chromosome. Upon brief contact between donor and recipient, DNA transfer occurs. After conjugation takes place, donor cells are eliminated with an antibiotic to which the recipient cells are resistant. Recipient cells containing the transferred plasmid are identified based on the selectable marker gene carried by the plasmid. One advantage of this method is that it does not rely on the development of procedures for protoplast formation and regeneration of cell wall. In addition, this method offers the possibility of bypassing restriction barriers by transferring single-stranded plasmid DNA. Introducing DNA by conjugation from donor *E. coli* has proven useful with *Streptomyces* and *Corynebacterium*.

12.3.2 Vectors

Selection of a cloning vector to carry out genetic modifications depends on the choice of the gene transfer method, the desired outcome of the modification and the application of the modified microorganism. Several classes of vectors exist and the choice of which to use must be made carefully. Replicating vectors of high or low copy numbers are commonly used to express the desired genes in heterologous hosts for manufacturing expressed proteins. Replicating vectors are also used to increase the dosage of the rate-limiting gene of a biosynthetic pathway such as that used for an amino acid, to enhance the production of the metabolite. Cosmid and bacterial artificial chromosome vectors which accept DNA fragments as large as 100 kb are necessary when cloning a large piece of DNA into a heterologous host for manipulation and high-level metabolite production. Conjugal vectors facilitate gene transfer from an easily manipulated organism such as *E. coli* into a desired organism that is usually more difficult to transform. Gene replacement vectors allow stable integration of the gene of interest. Food-grade vectors differ from the conventional cloning vectors in that they do not carry antibiotic resistance marker genes.

Special consideration must be given when constructing GMMs for industrial applications. If a GMM is to be released into the environment as a biological control agent, conjugal vectors should be avoided to prevent the horizontal transfer of the vectors and the genes

into indigenous microorganisms. If a GMM is used as a starter culture for food fermentation, conjugal vectors should also be avoided and food-grade vectors should be developed and used for genetic manipulation.

12.3.3 Promoters

A promoter is a segment of DNA that regulates the expression of the gene under its control. Constitutive promoters are continuously active; inducible promoters become activated only when certain conditions such as the presence of an inducer are met. It is important to select an appropriate promoter to optimize the expression of the target genes for desired timing and level of expression. A strong constitutive promoter is used when continuous expression of a target gene is desirable. For example, constitutive promoters are used to drive the expression of selectable marker genes to achieve complete elimination of non-transformed cells. However, inducible promoters are often chosen when it is necessary to control the timing of target gene expression. This is especially true when expressing foreign genes including toxin genes in *E. coli*.

The most commonly used inducible promoter for target gene expression in *E. coli* is the lac promoter and is turned on when the non-hydrolyzable lactose analog isopropylthiogalactopyranoside (IPTG) is added to the growing culture. This promoter is relatively weak and therefore is often suitable for expressing genes encoding toxic proteins. Promoters dependent on IPTG induction are usually undesirable for large-scale production of therapeutic proteins because of the high cost of the inducer and potential toxicity.

12.3.4 Selectable Marker Genes

Selectable marker genes which often encode proteins conferring resistance to antibiotics are an important part of cloning vectors and are required for identification of transformed cells. Application of selection pressure is necessary because the number of transformed cells is often significantly less than the number of non-transformed cells. Transformed cells are identified using a toxic concentration of the selection agent to inhibit the growth of the non-transformed cells. Usually, high-level expression of a selectable marker gene is necessary to ensure complete elimination of non-transformed cells.

Antibiotic resistance marker genes, although routinely used are not generally acceptable for the construction of recombinant organisms such as lactic acid bacteria and yeasts used for food fermentation. For lactic acid bacteria, alternative selection systems based on plasmid-linked properties of the organism itself including lactose metabolism,

proteolytic activity, DNA synthesis and bacteriocin resistance have been developed and incorporated into cloning vectors. One problem associated with these selection systems is that they tend to give more non-transformed background cells than the antibiotic resistance marker gene-based selection systems.

12.4 Strategies for Genetic Engineering of Microorganisms

Several strategies have been developed to create GMMs for desired traits. They include:
(1) Disruption or complete removal of the target gene or pathway.
(2) Overexpression of the target gene in its native host or in a heterologous host.
(3) Alteration of gene sequence and thereby the amino acid sequence.

12.4.1 Disruption of Undesirable Gene Functions

Disruption of a gene function can be achieved by cloning a DNA fragment internal to the target gene into a suitable vector. Upon introducing the recombinant plasmid into the host organism, the internal fragment of the gene along with the vector is integrated into the host chromosome via single-crossover recombination. The integration results in the formation of two incomplete copies of the same gene separated by the inserted vector sequence thereby disrupting the function of the target gene. However, such integration is unstable because of the presence of identical DNA sequences on either side of the vector. The recombinant strain often undergoes a second recombination that will “loop” out the recombinant plasmid from the chromosome thereby restoring the normal function of the target gene.

To create a stable recombinant strain blocked in the unwanted gene function, a gene replacement plasmid carrying two selectable marker genes is required. The first selectable marker gene originating from the cloning vector is used to select the transformed cells, whereas the second selectable marker gene is inserted into the target gene. The recombinant plasmid is introduced into the host organism followed by the selection of transformed cells based on the first selectable marker gene. Upon double-crossover recombination, the second selectable marker gene inserted into the target gene on the host chromosome disrupts the sequence of the target gene and destroys gene function. The recombinant strain is selected based on its resistance to the second selectable marker gene product and its sensitivity to the first selectable marker gene product.

Another approach to disrupting gene functions relies on antisense technology. The technology is based on antisense ribonucleic

acid (RNA) or DNA sequences that are complementary to the messenger RNAs (mRNAs) of the target genes. The binding of an antisense molecule to its complementary mRNA results in the formation of a duplex RNA structure. The activity of the target gene is inhibited by the duplex RNA structure because of:

(1) Inaccessible ribosomal-binding site that prevents translation.
(2) Rapid degradation of mRNA.
(3) Premature termination that prevents transcription.

Antisense technology has been used to downregulate target gene activities in bacteria. The main advantages of this approach are rapid implementation and simultaneous downregulation of multiple target genes. In addition, this method is ideal for downregulation of primary metabolic gene activities without creation of lethal events.

12.4.2 Overexpression of Desired Genes

High-level expression of a target gene may be achieved by employing a high copy number vector. Gene expression systems based on high copy number vectors have a number of drawbacks. One is the segregational instability of recombinant plasmids which results in the loss of recombinant plasmids and therefore loss of the desired traits. For example, expression of the *Bacillus thuringiensis* (Bt) toxin gene from a high copy number vector in *Pseudomonas fluorescens* was undetectable because of plasmid instability. Segregational instability of plasmids is usually resolved by maintaining recombinant strains under selective pressures usually by means of antibiotics. However, concerns about the use, release and horizontal transfer of antibiotic resistance marker genes suggests the need for development of other means to maintain the stability of plasmid.

Main concern about the use of high copy number vectors for high-level protein production in bacterial cells especially in *E. coli* lies with the formation of insoluble protein aggregates known as inclusion bodies. Inclusion bodies are biologically inactive because of protein misfolding which is a consequence of rapid intracellular protein accumulation. Although methods exists to isolate and renature inclusion bodies, these systems are often inefficient and steps need to be added up in the purification of active proteins. Also, in the process of renaturation of proteins, a significant percentage of the proteins remains denatured and inactive.

12.5 Improving Protein Properties

Site-directed mutagenesis and DNA shuffling are two powerful technologies that alter gene sequence in vitro to produce proteins that have improved characteristics. Site-directed mutagenesis is a technique used to change one or more specific nucleotides within a cloned gene to create an altered form of a protein via change in a specific amino acid. This technique has been used successfully to identify catalytically important residues in new proteins. Two examples include the identification of catalytically essential residues in the *Aspergillus oryzae* amylase A and the identification of the active site residue in the *Clostridium thermosulfurogenes*.

DNA shuffling, a technology introduced in 1994 is based on error-prone polymerase chain reaction and random recombination of DNA fragments. DNA shuffling may involve a single gene or multiple genes of the same family. Family gene shuffling is more powerful than shuffling of single genes because it takes advantage of the natural diversity that already exists within homologous genes.

Site-directed mutagenesis and DNA shuffling have been applied successfully for the improvement of numerous commercially important enzymes notably the enzymes used in laundry detergents. The goals sought commonly include altered substrate specificity, improved enzyme activity under broad washing conditions such as pH and temperature, enhanced resistance to detergent additives such as bleach and longer shelf life. To improve enzyme characteristics using site-directed mutagenesis, prior knowledge regarding the enzyme such as its active site and substrate-binding site is required.

The advantage of site-directed mutagenesis is that only a limited number of recombinants will be screened. DNA shuffling, on the other hand, does not require specific knowledge about the enzymes of interest and can create new variants containing multiple beneficial mutations in the gene sequence for maximum benefit. High-throughput screening assays for identifying desired recombinants are necessary for using this method.

12.6 CHECK YOUR PROGRESS QUESTIONS

1. What are inclusion bodies with respect to synthesis of transgenic protein in bacteria?
2. What is a promoter?
3. What are the three different strategies involved in the genetic engineering of a microorganism?

12.7 ANSWERS TO CHECK YOUR PROGRESS

1. Site-directed mutagenesis is a technique used to change one or more specific nucleotides within a cloned gene to create an altered form of a protein via change in a specific amino acid.
2. Several proteins such as insulin, interferons (IFNs) and interleukins are now produced by GMMs for therapeutic use. In addition, numerous vaccines for diseases (E.g., Hepatitis B) have been made possible by use of GMM's.
3. The different GMO crops that have *Bt* gene incorporated into them are: Corn, Tomato, Potato, Cotton, Brinjal., Sugarcane, *etc*. The restriction in the Indian scenario of GMO crops have been placed on the food crops that are directly consumed by the masses. The cash crops such as cotton are permitted to be cultivated.

12.8 SUMMARY

Development of recombinant deoxyribonucleic acid technology, the metabolic potentials of microorganisms are being explored and harnessed in a variety of new ways. Genetically modified microorganisms have found applications in human health, agriculture and bioremediation and in industries such as food, paper and textiles. Genetic engineering offers the advantages over traditional methods of increasing molecular diversity and improving chemical selectivity. This Unit provides the reader with information pertaining to genetically modified microorganisms and molecular tools for genetic engineering of microorganisms

12.9 KEYWORDS

Genetic Engineering, Genetically Modified Microorganisms, Microorganisms, Molecular Tools.

12.10 SELF ASSESSMENT QUESTIONS & EXCERCISES

1. What are the different molecular tools used in the genetic engineering of microorganisms?
Refer section 12.4.

2. Explain the different strategies used in the genetic engineering of microorganisms to express the gene of interest.
Refer section 12.5

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UNIT-XIII
APPLICATION OF GMM - DERIVED PRODUCTS

Structure

13.1 INTRODUCTION

Recombinant DNA technology uses genetic engineering to introduce genes into microorganisms and other cells. They help produce metabolites of commercial or medical importance such as insulin, vitamins, amino acids or enzymes. Using microorganisms is much environmentally friendly than conventional chemical synthetic method as they use less energy and use renewable resources. Today, genetically modified microorganisms (GMMs) have found applications in human health, agriculture and bioremediation.

13.2 OBJECTIVES

This unit gives detailed information on the applications of Genetically Modified Microorganisms in human health, Agriculture and Environment.

13.3 GMMS IN HUMAN HEALTH

13.3.1 Recombinant Therapeutic Proteins

Several proteins such as insulin, interferons (IFNs) and interleukins are now produced by GMMs for therapeutic use. The traditional method of supplying these proteins to patients requires purification of the proteins from cells, tissues or organs of humans, cows or pigs. Because it was impractical to treat diabetes with human insulin from cadaver sources, cow and pig insulin which are somewhat different from human insulin were substituted. The problems with obtaining the proteins directly from animal sources included the limited supply and potential immunological responses. Limited supply translates into higher cost for the medication.

These problems can sometimes be avoided by producing the proteins in microorganisms. Human insulin, the first recombinant therapeutic protein approved by the Food and Drug Administration (FDA) in 1982 was produced by genetically engineered *E. coli* containing the human insulin genes. Human growth hormone approved by the FDA in 1985 was produced by a modified *E. coli* strain containing the native human growth hormone gene.

13.3.2 Recombinant Vaccine

Hepatitis B is a serious disease caused by hepatitis B virus that attacks the liver. The first vaccine against hepatitis B was prepared with the purified hepatitis B surface antigen (HBsAg) extracted from blood samples of infected individuals. This process is unsafe because of the risk in handling the infectious agent and expensive because of the required animal testing. In addition, the vaccine may be contaminated with other infectious agents. The second generation of hepatitis B vaccine was produced by expressing the gene coding for hepatitis B surface antigen in Saccharomyces cerevisiae, common baker's yeast.

13.4 Animal Health

13.4.1 Recombinant Proteins

Proteins benefiting animal health are also produced by recombinant microorganisms. Bovine somatotropin (bST), a natural protein hormone produced in the pituitary glands of cattle regulates both animal growth and milk production in lactating dairy cows. Injection of pituitary extracts into lactating cows boosts milk production. However, pituitary glands from as many as 25 cows are needed to provide sufficient bST to supplement 1 cow for 1 day.

13.4.2 Recombinant Vaccine

Rabies, a viral disease encountered by humans and other mammals leads to more than 35,000 human deaths and several million animal deaths worldwide every year. The rabies virus reservoir is primarily in wild animals including fox, skunks, raccoons, wolves, mongooses and dogs. Humans normally become infected with the virus through bites from infected animals. Upon exposure to the virus, the current method to prevent development of rabies in humans and domestic animals is to inoculate with rabies vaccine prepared from an attenuated strain of rabies virus. This is normally in addition to treatment using antirabies γ globulin. However, this method is impractical to eradicate rabies in wild animals.

13.5 Textile Industry

Microbial enzymes have been used in the textile industry since the early 1900s. To commercialize the enzymes, they must be produced at high levels. Conventional methods to enhance production include optimizing medium composition, growth conditions, andthe fermentation process. Random mutagenesis and screening arecommonly used to achieve high yields. Genetic engineering offers a possibility in which high-level enzyme production is achieved in a heterologous host to overcome the limitations of the natural producing organism. Two examples are amylase of *Bacillus stearothermophilus* and cellulase of the alkaliphilic Bacillus BCE103. Amylases have been used for many years to remove starch sizes from fabrics, known as de-sizing. Originally, amylases from plant or animal sources were used. Later, they were replaced by amylases of bacterial origin. Cellulases prevent and remove fuzz and provide color brightening of cellulose-based fabrics such as cotton.

13.6 Food Industry

Enzymes manufactured by GMMs have been used in the food industry for more than 15 years. Well-known examples include the use of chymosin for cheese making and pectinases for fruit and beverage processing. Traditionally, cheese making requires chymosin-containing rennet from calf stomachs to provide the essential proteolytic activity for coagulation of milk proteins. However, chymosin preparations could have animal sources of contaminants.

Production of pectinases *via*. the genetic engineering approach focuses on economic enzyme production, enhanced enzyme purity, and environmentally friendly production processes. Complete degradation of pectin, a natural substance found in all fruits, is important for the

beverage industry. The reason is that complete degradation of pectin increases juice extraction from fruits, enhances juice clarification, and helps the filtration step of the process.

13.7 Diagnostic Tools

Acquired immunodeficiency syndrome (AIDS) immunological tests are used for diagnosing the disease and for testing donated blood samples. The first generation of AIDS tests, commercialized in 1985, was based on inactivated human immunodeficiency virus (HIV) grown in tissue culture. This production method is both expensive and, more importantly, hazardous because of the risk from handling the infectious agent. Further, this first-generation AIDS test was subject to false-positive reactions because of the cellular debris from virus-producing human cells. These problems were overcome by cloning the gene encoding the relevant antigenic coat protein of the virus into E. coli for large-scale production of the protein.

13.8 Biodegradable Plastics

Conventionally, plastics polymers are made via petroleum-based processes. Because of the growing concerns over the environmental impact of petroleum-derived polymers, alternative methods to synthesize the polymers are under investigation. Many microorganisms naturally produce polyhydroxyalkanoates (PHAs) in the form of granules that the organisms use as an energy storage material. PHAs are genuine polyester thermoplastics with properties similar to the petroleum-derived polymers. In addition, PHAs are degradable by depolymerase, an enzyme family widely distributed among bacteria and fungi. These characteristics make PHAs an attractive replacement for the petroleum-based polymers. However, the microorganisms that naturally produce PHAs are not necessarily suitable for commercial PHA production, mostly because of slow growth and low yields.

13.9 In Agriculture

13.9.1Biological Control of Frost Injury in Plants

Frost damage is a major agricultural problem affecting many annual crops, deciduous fruit trees, and subtropical plants. In addition to the losses caused by frost injury, hundreds of millions of dollars are spent every year to reduce plant frost injury mechanically. These methods are both costly and ineffective.

Frost damage is initiated by bacteria belonging to the genera Pseudomonas, *Xanthomonas* and *Erwinia* collectively called ice-nucleating bacteria. The bacteria, living on the surface of the plants, possess a membrane protein that acts as an ice nucleus for initiation of ice crystal formation. Ice crystals disrupt plant cell membranes, thus causing cell damage. The biological route of controlling the nucleating bacteria is through seed or foliar applications of non-ice-nucleating bacteria to outcompete ice-nucleating bacteria. The non-ice-nucleating bacteria were isolated by treating the ice-nucleating bacteria with chemical mutagens.

13.9.2 Biological Control of Insect Pests

BT, a naturally occurring soil-borne bacterium, produces unique crystal-like proteins that have larvicidal activities against different insect species and pose no harm to mammals, birds, or fish. The crystal-like proteins bind to specific receptors on the intestinal lining of susceptible insects, causing the cells to rupture. Because of these unique features, Bt has been used as a safe alternative to chemical pesticides for several decades. However, natural Bt-based products do possess some shortcomings, including instability in the natural environment, narrow host range, need for multiple applications, and difficulty in reaching the crop's internal regions where larvae feed.

13.9.3 Biological Control of Plant Disease

Plant pathogens, including fungi and bacteria, damage crops and thereby reduce crop yield. Plant diseases are conventionally fought with chemicals, a strategy that is expensive, inconvenient, potentially environmentally unfriendly, and sometimes ineffective. An alternative method is to develop biological control agents in which microorganisms are modified to deliver the desired chemicals.

Agrobacterium tumefaciens causes crown gall disease in a wide range of broad-leaved plants by transferring part of its DNA (T-DNA), located on a large tumor-inducing (Ti) plasmid into the plant cell. Upon integration of T-DNA into the plant host's chromosome, the genes on T-DNA are expressed, resulting in overproduction of plant growth hormones and opines. Overproduction of plant growth hormones causes cancerous growth, whereas opines are believed to serve as nutrients for the bacterium.

13.10 In Environment

Bioremediation

Bioremediation refers to the utilization of biological systems to detoxify environments contaminated with heavy metals such as mercury and lead, organic compounds such as petroleum hydrocarbons, radionuclides such as plutonium and uranium, and other compounds, including explosives, pesticides, and plastics. The first field release of a GMM for bioremediation was *Pseudomonas fluorescens* HK44 for naphthalene degradation. Strain HK44 was derived from *P. fluorescens* isolated from a site heavily contaminated with polyaromatic hydrocarbons. HK44 contains a plasmid capable of naphthalene catabolism. In addition, this genetically modified strain harbors a bioluminescence-producing reporter gene (lux) fused with the promoter that controls the naphthalene catabolic genes. Therefore, in the presence of naphthalene, the naphthalene genes are expressed, resulting in naphthalene degradation and emission of luminescence from the recombinant strain. The presence of the reporter system facilitates real-time monitoring of the bioremediation processes.

13.11 CHECK YOUR PROGRESS QUESTIONS

1. Write on the application of genetically modified organisms on human health.

2. Describe the application of genetically modified organisms on Animal health.

3. What are the applications of genetically modified organisms in Agriculture?

13.12 ANSWERS TO CHECK YOUR PROGRESS

1. Refer section 13.4.
2. Refer section 13.5.
3. Refer section 13.10.

13.13 SUMMARY

Recombinant DNA technology uses genetic engineering to introduce genes into microorganisms and other cells. They help produce metabolites of commercial or medical importance such as insulin, vitamins, amino acids or enzymes. Using microorganisms is much environmentally friendly than conventional chemical synthetic method as they use less energy and use renewable resources. There are a variety of applications for genetically modified microorganisms in the fields of therapeutics, agriculture, animal health, textile industries and in the process of bioremediation.

13.14 KEYWORDS

Animal health, Applications, Environment, Genetically modified organisms, Human health, Industries

13.15 SELF ASSESSMENT QUESTIONS & EXCERCISES

1. What is Site-directed Mutagenesis?
2. What are some of the products that are produced by GMM's that are useful in human health?
3. What are the different GMO crops that have been incorporated with *Bt* gene? Why are specific *Bt* crops restricted for used in India?

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UNIT-XIV

ETHICAL ISSUES RAISED BY GMO

Structure

14.1 INTRODUCTION

Unlike experiments carried out on humans and animals, applications involving microorganisms and their genetic modification do not cause pain and suffering as generally understood. The ethical problems raised by genetically modified microorganisms (GMMs) therefore are mainly related to the impact they have on the earth's biosphere. As microorganisms can be found everywhere including in the ecological niches that are the most unsuited to life forms, the repercussions can be substantial and irreversible.

In order to clearly grasp to what extent GMMs can be dangerous for humans and their environment it is necessary to define them and explain the context in which they are used or can be potentially used and also to specify the possible risks of these uses along with the actual usefulness of these GMMs. The most effective way of solving this ethical problem once and for all is perhaps to reduce their use. The role of researchers in the emergence of these new organisms is that they cannot yet be completely controlled.

14.2 OBJECTIVES

This unit gives detailed information on the ethical issues raised by Genetically Modified Microorganisms.

14.3 GENETICALLY MODIFIED ORGANISMS

In a phylogenetic tree featuring all living organisms, the majority of these in terms of diversity are microorganisms where one branch of this tree includes true bacteria (also known as eubacteria) whereas the other two branches consists of archaebacteria and eukaryotic microorganisms. These notably include protozoa, unicellular algae, yeasts and moulds. Within these three main categories of microorganisms, it is bacteria that have undergone the most genetic modification. However, within the eukaryotes, moulds and yeasts have also undergone genetic modification, although this is rarer.

A microorganism is considered to be genetically modified if the genetic material has been inserted into it by an unnatural method other than natural conjugation, transformation or transduction. It may be also one in which the genetic material that has been modified in vitro has been inserted into it. Even if a natural transfer method has been used, the microorganism is still considered to be genetically modified.

The gene that has been inserted into the bacteria can be homologous (from the same species) or heterologous (from another species or bacterial genus - cloning of a *Bacillus thuringiensis* gene encoding an insecticide in Pseudomonas). A heterologous gene can also come from an animal or plant cell (cloning the gene encoding human insulin in *Escherichia coli*).

DNA modified in vitro (foreign DNA) can be either inserted into a cloning vector (a set of genes) capable of enabling the genes that have been transferred to the bacteria to replicate in it. In this case, the foreign DNA includes the cloned gene and more often the heterologous genes making up the cloning vector or inserted into the chromosome (the foreign DNA is generally exclusively made up of the cloned gene).

14.4 Usage of Genetically Modified Microorganisms

There are five categories in which microorganisms or potentially including GMOs can be used:

a) Producing molecules or biomass in a fermenter
b) Producing fermented foods
c) Various uses in the environment (in agriculture, for pollution control *etc.*)
d) Producing strains for therapeutic purposes (*e.g.* live vaccines)

e) Gaining fundamental knowledge

With certain technologies, living microorganisms can be released into the environment where they may multiply. To guard against this risk, many countries have legislated against these GM technologies.

14.5 Usage of Microorganisms in a Fermenter

Since the mid-20th Century, microorganisms have been widely used to produce numerous molecules required by the pharmaceutical, agri-food and chemical industries. Moreover, they are produced in fermenters to develop the starter cultures required by the fermented food industry.

In the pharmaceutical industry, many molecules such as antibiotics or vitamin B_{12} are produced by microorganisms which synthesize them naturally. There are also more numerous molecules whose gene has been cloned in a microorganism (*e.g.* human insulin, growth hormone, Hepatitis B vaccine). All these molecules have been marketed for many years and are part of developed countries to be used for either daily or therapeutic arsenal. In the agri-food industry, intermediate products such as enzymes (*e.g.* amylases, rennin), amino acids, organic acids and even nucleic acids are produced by microorganisms. In the same way as above, these molecules can be naturally synthesized by microorganisms or come from GMMs. The chemical industry produces many molecules such as enzymes, organic acids and biofuels using microorganisms.

Producing Molecules

Producing molecules in a fermenter involves culturing the microorganism responsible for producing the required molecule in a generally confined space containing a suitable nutritive medium. This operation is generally performed in a confined atmosphere and in theory does not cause microorganisms to be released into the environment. There is little diversity in microbial species that are genetically modified to produce useful molecules. They are often model microorganisms that have been studied over a long period and for which the recombinant DNA technologies are very sophisticated and well-known. They include mainly bacteria (*E. coli*, *Bacillus subtilis*) and yeasts (*Saccharomyces cerevisiae*).

Producing Fermented Foods

Microorganisms are the main agents in the production of vast numbers of fermented foodstuffs. Bread, wine, cheese, butter, creme fraiche, yoghurts, kefir, fermented meats (dry-cured sausage, salami) and fermented vegetables (sauerkraut, olives) are produced by the

action of an extremely varied microbial flora. Some fermented foods are produced from a complex and little known microbial flora generally categorized as wild flora found in raw materials and the environment (some unpasteurized cheeses, beers and sourdough bread); others are made from industrial starter cultures of simpler composition and identified flora (many cheeses made from pasteurized milk) and lastly other fermented foodstuffs contain both complex wild flora and industrial flora which has been added deliberately and in controlledconditions especially for the fermented foodstuffs whose raw material cannot be sterilized such as dry-cured sausage, *etc*.

Whatever the case, apart from the majority of fermented drinks and bread, at the end of fermentation, the fermented foods generally contain roughly 10^9 microorganisms/gram. Depending on the type of food, this flora may or may not be living when the food is ingested by the consumer. Microbial flora then travels through the digestive tract and does not generally stay there consequently ending up in the environment through faeces.

Usage of GMOs for Fermented Foods

Foods with complex flora are useful due to their elaborate organoleptic properties but they can be the seat of pathogenic microorganism development. In addition, their production is difficult to control as microbial flora fluctuates according to the environmental conditions. On the other hand, foods with controlled flora are less useful from an organoleptic viewpoint but safer. For several years, in order to produce fermented products with controlled flora that are as advantageous from an organoleptic viewpoint as wild flora-derived foods, industrialists have added new bacterial species isolated from wild flora-derived foods to their starter cultures. But until very recently, they were unable to guarantee that these new species were totally harmless which thus raised doubts about the safety of these foods. The use of GMMs to produce fermented foods would on the one hand solve the problem related to the use of little-known flora and on the other hand overcome the various technological problems with the development of improved strains.

Usage of Microorganisms for various applications in the Environment

Microorganisms are notable for many pollution control processes, the most common of which is sewage treatment, a process that involves highly complex wild flora. Methods for controlling pollution of more specific compounds (hydrocarbons, slurry, various

pesticides, *etc*.) has also been developed and involve selected flora which is less complex in terms of diversity. However, the action of this flora is far from optimal and therefore requires genetic improvement. Numerous microbial GMOs with properties that are compatible with the process of rendering resistance to the substrate to be biodegraded, good establishment in the environment, *etc*. have been developed in laboratories but they cannot be used for legal reasons as there is a risk of uncontrolled dispersal into the environment.

In agriculture, microbial strains are used to enhance the growth of plants and crop protection. It has been necessary to develop genetically recombinant strains to optimize the processes. Strains of *Sinorhizobium meliloti* that have been genetically improved to enable nitrogen fixation by the plant have been used since 1997 to seed legume crops. Similarly, pesticides using other genetically improved species (*Agrobacterium radiobacter*) are used in soils.

Strain Production for Therapeutic Purposes

Owing to their ability to survive or pass through human and animal mucosa, microorganisms can be used to treat or prevent certain diseases. For example, a strain of *Lactobacillus jensenii* has been modified to secrete the CD4 protein used by the HIV virus in the vaginal mucosa to penetrate lymphocytes. Even if these GMMs can solve major therapeutic problems they are not always used because of their potential dispersal into the environment.

Use of Microorganisms in Laboratories for Knowledge Upgradation

Genetically modified microorganisms in research laboratories enable us to better understand how microorganisms function. Numerous genes belonging to a wide variety of microbial species have therefore been cloned and have given rise to thousands of GMM strains used as research material by researchers.

14.6 Risks posed by Genetically Modified Microorganisms

The unicellular nature and relative simplicity of microorganisms refers their ableness to multiply very rapidly: *Escherichia coli* is therefore able to give birth to two new cells in 20 minutes when it is cultured in an optimum culture medium. In the same way, *Lactococcus lactis* divides in 30 minutes when it is cultured in milk for the production of soft cheeses. This property together with their extremely

small size indicates that they are able to be dispersed efficiently into the environment by wind and water, allowing microorganisms to reach a great variety of potential ecological niches. The ease with which they are able to colonize these ecological niches allows the microorganisms to adapt to a wide variety of environments which may pose difficulties for any other living organism. These bacteria demonstrate an extreme genetic adaptability due to the fact that they are haploid (contain one chromosome meaning that any mutation is clearly expressed) and due to the fact that they may acquire genes from other microorganisms by horizontal transfers. These mutations and acquisitions of new genes give rise to the appearance of a large number of microbial variants which are selected through pressure from the natural environment if the characteristics that they have acquired allow them to adapt more successfully to this environment.

The danger posed by these genetically modified organisms is therefore related both to their dispersal into the environment and to their potential for adaptation to a new environment whereby altering the animal and plant microbial ecological balance causing disruption in the environment to a greater or lesser extent. If unable to develop in the environment, they may potentially transfer their modified genetic material to other microorganisms thus leading to the appearance of new variants which themselves may have the capability of greatly disrupting the environment.

14.6.1 Methods of Gene Transfer

Microorganisms are capable of acquiring new genes from other living microorganisms or microbial corpses in the natural environment. In the latter case, the free DNA in the environment is often damaged, although some habitats such as marine sediments limit this damage. There are three main types of mechanisms for gene transfer between microorganisms: conjugation, transformation and transduction.

Conjugation is a form of gene transfer between two bacterial cells. Certain genes known as “transfer genes” bring cells closer together and through the formation of a cytoplasmic bridge allow DNA to travel between the two bacteria (donor and recipient). In general, transfer genes are carried by plasmids (small loops of DNA which are capable of replication independently of the chromosome) which replicate both in the donor and the recipient bacteria. These plasmids do not only contain transfer genes but also other genes which often allow the bacteria to adapt to an ecological niche (genes encoding numerous resistance mechanisms or allowing the assimilation of nutritional elements). Bacteria having acquired a plasmid by conjugation may in turn transfer it thereby acquiring new properties. However, these plasmids known as conjugative plasmids are not able to be transferred

to all bacterial species. There is a host specificity whereby some plasmids are able to replicate and be expressed in species which are very varied from a phylogenetic point of view while others have a narrow host spectrum which only allows them to replicate and be expressed in a small number of species or even just within a single bacterial species. This host spectrum controls and limits conjugative transfer in the bacterial world. In the case of GMMs, some genetic improvements are relatively frequently encoded and carried by a plasmid known as a cloning vector. In order to limit gene transfers, these vectors do not carry transfer genes. Nevertheless, it is possible that they may be transferred to other bacteria if they are accompanied by a conjugative plasmid. During conjugation, plasmids which do not possess transfer genes may benefit from the enzymatic machinery established by the conjugative plasmid in order to cross into the recipient bacteria at the same time as the conjugative plasmid. However, vectors which are transferred in this way must then be capable of replicating and being expressed in the recipient bacteria.

Transformation is a mechanism which allows some bacteria to acquire exogenous DNA from dead and lysed cells which circulates freely in the natural environment to integrate it into its genome. This mechanism which has been very well described for some bacterial species allows bacteria to repair their genome when it is damaged by exchanging damaged genes with others from dead bacteria and also to acquire new genes from other bacteria. In the same way this mechanism does not allow bacteria to acquire just any type of gene. The DNA which penetrates the bacteria must be able to be incorporated into the genome and must therefore have a certain sequence homology in order to allow recombination with the chromosome. Moreover, this mechanism may be more or less effective depending on the species and it is not yet known whether all species possess this mechanism or one of a similar nature.

Transduction is the transfer of bacterial DNA to other bacteria by a bacteriophage, a virus specific to bacteria. When bacteria is infected by a bacteriophage, an event which occurs frequently in nature is that this virus when it multiplies in the bacterial cell carries with it bacterial DNA instead of phagic DNA. When it reinfects new bacteria, it therefore injects it with this bacterial DNA. If it is plasmid DNA, it may potentially be able to survive in the bacteria. If it is chromosomal DNA, it may potentially become incorporated into the chromosome in so far as it is sufficiently homologous for a recombination to occur. For bacterial GMOs, the transfer of modified DNA may well take place by transduction; the capacity of the cloning vector to replicate in the recipient bacteria depends on its host spectrum and in the case of chromosomal DNA, the ability to incorporate into the chromosome

depends on the degree of homology with the DNA of the recipient bacteria.

These gene transfer mechanisms may allow a diverse flora to acquire new properties. This means that a genetically modified microorganism has the potential to transfer dead or alive genes which have been modified in laboratories. The unregulated acquisition of new genes by microorganisms may potentially lead to the appearance of new pathogenic germs (for example through the acquisition of genes involved in virulence or in the colonization of a new organism). These gene transfers may also lead to unwanted biochemical reactions which may have an impact on the environment damaging important plant compounds or to the production of toxic compounds.

It is also conceivable that these gene transfers may lead to the emergence of new microbial variants which may acquire properties allowing them to colonize a new ecological niche and to replace the endogenous population thus disrupting the microbial balance.Although gene transfers between microorganisms are frequent and these mechanisms are partially identified, they are nevertheless a cause for concern in the case of GMMs. For example, researchers have shown that gene transfers between *Agrobacterium tumefaciens*, the bacteria used to modify the genes of plant species and *Pseudomonas fluorescens* most likely occur naturally in soils. Gene transfers between microorganisms and so-called "superior" organisms are less well-known, but nonetheless probably take place.

14.8 Development of New Microorganisms in the Environment

Genetically modified microorganisms are often designed for a specific function. Nevertheless, we do not have sufficient knowledge of the microbial metabolic pathways in order to have a complete control over the appearance of new properties resulting from the modification of genes in laboratories. If unforeseen metabolic modifications can occur in a microorganism whose genome has been modified, it is also highly likely that they can occur in other species in the environment which have acquired these modified genes through horizontal transfer.

It should be remembered at this stage that microorganisms exist in huge numbers in the environment (for example, there are at least 109 microorganisms per gram in fertile humus, 105 per milliliter in the water of a river running through a town and 109 per milliliter at the outlet of a sewage treatment plant) and they are represented by a large number of diverse species (there are several hundred in the digestive tract of mammals, for example, or in the anaerobic reactor of a sewage treatment plant). The possibilities for gene transfers between these

microorganisms in a given ecosystem are therefore numerous and may lead to the emergence of more adapted variants.

If these new properties give a selective advantage to a species, it is possible that this species being better adapted to a new environment may colonize it thus greatly disrupting the ecological balance whether microbial, plant or animal. Such a problem is genuinely conceivable and was apparent even before the arrival of GMMs. Some cases are already known in which microorganisms have found themselves in a new ecological niche as a result of human intervention. They have subsequently colonized this niche, disrupting it to a great extent. A well-known example of this involves the toxigenic unicellular alga, *Chrysochromulina polylepis* which because of human activity (the release of nitrogenous substances into the sea) invaded part of the North Sea and the English Channel leading to significant health problems as it produces toxins which are pathogenic for humans. Even though not a microorganism, this is also the case of the alga *Caulerpa taxifolia* which probably as a result of the accidental emptying of the aquariums of the Oceanographic Museum in Monaco is currently colonizing the Mediterranean Sea bed causing the death of the endogenous fauna.

14.8.1 Multiplication of Pathogenic Microorganisms

Research into pathogenic microorganisms requires the cloning of genes encoding the pathogenicity factors in other microbial species, thereby giving rise to genetic events which would probably not naturally have taken place and therefore the appearance of strains representing a new pathogenic power. Even though these laboratory experiments concerning plant, animal and human sectors do not directly aim to create strains which are more pathogenic but to aid the understanding of pathogenicity mechanisms, our current level of knowledge means that we are not able to ensure the complete harmlessness of these transgenic microorganisms. GMOs threaten to escape the control of scientists and to have unpredictable consequences on animal and human species.

14.8.2 Natural Genetic Modifications of Microorganisms

Nature does not need humans in order to modify the genetic inheritance of microorganisms. Their extremely short generation time compared with that of superior beings such as animals which allows them to reach a very high population size in a short space of time means that microbial populations continuously generate a large number of mutants. A mutant may be defined as an individual which has a different genetic inheritance from that of its parents without having acquired new genes by horizontal transfer. A mutation may be a one-off variation in a nucleotide base within a genome or a genetic rearrangement (gene inversion, loss or duplication).

The phenomena which lead to mutations may be natural or caused by external agents of the natural environment. Some bases which make up genes may thus naturally change and be transformed into other bases. Elements from the external environment such as radiation (ultraviolet rays) or chemical molecules resulting from human activity or the activity of other organisms including microorganisms may be responsible for these mutations.

Such mutations are actually due to lesions in the genetic material which if poorly repaired give rise to a change in genetic information. The environment will then carry out a process of selection allowing the emergence of the best adapted mutants. Consequently, when someone suffering from a bacterial infection is treated with an antibiotic the use of the antibiotic may inadvertently lead to the selection of mutants which are resistant to it. These few mutants which are resistant to the antibiotic and which constitute just a small proportion of the infecting bacterial population will thus survive and replace all the original population.

This appearance of mutants with new properties is a phenomenon which has been well known for some time. Spontaneous yeast mutants have thus been used in brewing techniques for several decades. As it is a natural and commonplace occurrence, mutagenesis is frequently used to improve the properties of microorganisms in all sectors where they are used (the pharmaceutical and food- processing industries, farming, pollution control, *etc*.). Strains modified in this way may be used and then released/ used in the environment without posing any problems, ethical or otherwise.

The extreme diversity of microbial species and the large populations mean that genetic transfers between individuals are likely to be considerable in nature, thus contributing greatly to microorganism's exceptional capacity for adaptation to environmental variations. If humans are able to carry out genetic modifications of microorganisms by the introduction of new genes, it should be remembered that this process also occurs naturally in the environment.

14.8.3 Non-adaptability of GMO

During their time in vitro, "laboratory creatures" have a tendency to lose their capacity to colonize an environment or even to survive in their natural habitat. Several experiments have demonstrated that once the model strains which are used in laboratories (*Escherichia coli*, *Saccharomyces cerivisae*, *Bacillus subtilis etc*.) are removed from their test tubes, they have very little chance of surviving in their natural habitat. Successive culture on rich or selective media of these model

strains used in laboratories or genetic modifications carried out by researchers mean that they have long since lost some of their nature which are necessary for their adaptation to a complex environment colonized by a diverse flora. This observation does not just concern model species as, even when a strain taken from the natural environment and thus adapted to a complex flora is isolated and is cultivated for just a short time in a laboratory; it rapidly loses the facilities which allow it to settle back into the environment from which it comes. Nevertheless, these microorganisms, even if unable to survive in their natural habitat are able on their death and subsequent cellular lysis to release DNA which may be captured by other microorganisms.

14.8.4 Advantages of GMO

Microbial GMOs may potentially be used in the manufacture of recombinant molecules (drugs, intermediate products in the agri-food industry), the production of fermented foodstuffs (alcoholic drinks, dairy products, *etc.*), pollution control (sewage, hydrocarbons), the improvement of crop production by facilitating symbiosis between plants and microorganisms (nitrogen fixation) and the substitution of various technologies (biolixiviation, *etc.*).

The reasons which are leading to the use of GMMs or which may do so in the future may be of an economic nature and/or may relate to the health of humans, animals or plant life. Every time a GMM is used, it is imperative that the risks involved in its use be compared to those which would exist if the GMM were not used.

In theory, in the case of molecules produced in fermenters, the GMM is not released into the natural environment. In order to ensure that there are no leaks, fermentation plants must be made more secure, therefore adding to the costs involved in the process. Nevertheless, this process is controllable and therefore considerably reduces the risks involved in the use of GMMs.

`Some of the molecules produced in this way have enabled the treatment of conditions for which the pharmaceutical alternatives were far from comparable and even represented acertain risk. For example, before it was produced using a recombinant *E. coli* strain, human growth hormone was produced using hypophyses taken from human corpses. Some batches which were made from hypophyses contaminated with the agent responsible for Creutzfeldt- Jakob disease was responsible for the transmission of this disease to children being treated for dwarfism with this hormone. Before recombinant insulin became commercially available, diabetics were treated with modified pig insulin against which after a certain period of time, the human body produced antibodies.

In addition to these important advantages concerning the purity and quality of the molecules such cloning of microorganisms offers due to the ease of production, the two-fold benefit of a considerable reduction in production costs allowing more people to be treated and the possibility of carrying out research into new uses. For example, growth hormone now has other applications which are well-known in the field of competitive sport over and above the restoration of growth in children suffering from dwarfism.

As far as the manufacture of drugs is concerned there seems to be no doubt that the use of GMMs insofar as they are not released into nature represents progress for our society. On the other hand, several other molecules are produced to essentially economic ends in order to improve or stabilize a technological process. This is the case for many enzymes which are used in food processing such as amylases which are used in brewing, proteases used in the meat industry and chymosin used during cheese production. There are often alternatives to the use of these molecules: they may be naturally synthesized by a microorganism which does not necessarily need to be a GMM (for example, *Bacillus subtilis* produces proteases) or they may be extracted from another organism (chymosin may also be extracted from calf rennet which has traditionally been used for centuries in cheese-making technology). In the eyes of their critics, the use of GMOs in the production of molecules may be seen to be questionable if these can be produced by an alternative process. It must be borne in mind, however, that the process in question - cultivation in fermenters - does not lead, in theory to the release of modified microorganisms into the environment. Moreover, the reduction in costs of a process through the use of molecules derived from GMMs may allow more people to consume the resulting food products.

As regards GMMs which are released or used in the environment, the risks involved in their use are greater than in the above example as it is conceivable that they may disrupt ecosystems, either because they may transfer their modified genes to another microorganism, providing the latter with “colonizing” properties or because their development may be to the detriment of endogenous species.

As far as GMMs which are available for use in the production of fermented foodstuffs are concerned these essentially aim to improve production processes (the development of strains of *L. lactis* which are resistant to bacteriophages for the production of cheeses is a good example) or to improve the organoleptic, nutritional and hygienic properties of food. These improvements may, however be achieved

using other means such as the selection of appropriate non-modified strains or adherence to hygiene standards.

The issues surrounding GMMs which are used in conjunction or in symbiosis with plants are more delicate. In this context, GMMs are used to improve plant productivity or to develop the resistance of crops to harmful insects or pesticides. A solution which allows production costs to be lowered or productivity to be improved especially in areas where crop production is difficult cannot be immediately rejected out of hand. Even if alternative methods of cultivation such as those which avoid the use of polluting fertilizers or pesticides or sustainable development appear to be less effective than the development of techniques which use GMMs it becomes difficult to justify rejection of these techniques.

The most complex issue relates to the use of live GMMs to treat certain human and animal diseases. For example, species used in food production such as *Lactococcus lactis* have been genetically modified in such a way as to secrete molecules through the mucosa (the digestive membrane, for example) in order to treat medical conditions. This form of treatment seems promising and should in some cases prove to be more effective than traditional allopathic medicine. These strains are ingested and passed through the human digestive tract and are therefore released in living form into the environment. This is why a current line of research involves the development of strains which will not be able to survive outside the digestive tract. In the meantime, however, if we compare the benefits, they provide with the potential risks posed to the environment it seems difficult at the current time to decide on whether these GMMs are absolutely necessary.

The final category which concerns GMMs produced in laboratories is by far the most extensive in terms of the diversity of the experiments carried out and the strains obtained. The cloning of microbial genes in model microorganisms such as *E. coli*, *B. subtilis* or *L. lactis* which is often necessary in order to obtain data on their regulation and expression is a procedure which is often very difficult to avoid. This is why the freezers of molecular biology laboratories are often overflowing with genetically modified strains. An understanding of the prokaryotic genome has often been a prerequisite for understanding more complex genomes such as the human genome which is why it seems impossible to consider the abandonment of research using GMMs insofar as the provisions set in place to avoid their dispersal into the environment are carefully followed.

14.9 Evaluation of the Legitimacy

As we have seen, fundamental research on genomes and on the way in which organisms work requires the use of genetic engineering meaning that the development of GMOs in laboratories does not always directly comply with sometimes arguable economic imperatives. However, experience has taught us that these scientific advances also serve interests which go against the well-being of humans and their environment. Nonetheless, it seems impossible to determine beforehand the consequences linked to the acquisition of new scientific knowledge which will be harmful to humankind.

It is indisputable that the acquisition of this fundamental knowledge is essential as it subsequently leads to applied research which aims in particular to improve the conditions in which humans live and to preserve their environment. Nevertheless, researchers are entitled to call into question some lines of research which may increase risks to the health of humans, animals or plant life or to the ecological balance of the planet. The development of microbial strains with improved pathogenic capabilities in order to expand the arsenal of biological weapons provides a clear illustration of this. Even though it may appear difficult at first sight, every researcher has a responsibility to be concerned about the potential consequences of his or her work.

The freedom of researchers confined to the purpose of their research is however becoming hindered to an ever greater extent by economic imperatives: in order to work, they need financial assistance which is dependent on the scientific guidelines dictated by the State or on the needs of businesses. If it can be said that the business community is directly motivated by economic imperatives and pays relatively little attention to humanitarian considerations this is not necessarily true for the State. However, over the past few years in France, the State has tended to withdraw from the field of research thus strengthening the ties between businesses and researchers.

14.10 CHECK YOUR PROGRESS QUESTIONS

1. Define Conjugation
2. Mention any two advantages of GMO
3. Comment on the risk posed by GMO

14.11ANSWERS TO CHECK YOUR PROGRESS

1. Transfer of genetical material between two bacterial cells through the formation of cytoplasmic bridge.
2. Refer 14.8.4
3. Refer 14.6

14.12 SUMMARY

A microorganism is considered to be genetically modified if the genetic material has been inserted into it by an unnatural method other than natural conjugation, transformation or transduction. It may be also one in which the genetic material that has been modified in vitro has been inserted into it. Even if a natural transfer method has been used, the microorganism is still considered to be genetically modified. Unlike experiments carried out on humans and animals, applications involving microorganisms and their genetic modification do not cause pain and suffering as generally understood. The ethical problems raised by genetically modified microorganisms (GMMs) therefore are mainly related to the impact they have on the earth's biosphere. As microorganisms can be found everywhere including in the ecological niches that are the most unsuited to life forms, the repercussions can be substantial and irreversible.

14.13 KEYWORDS

Application genetically modified organisms, Ecosystem disruption, Environment, Ethical issues, Gene transfer, Genetically modified organisms

14.14 SELF ASSESSMENT QUESTIONS & EXCERCISES

1. How are microorganisms used in the fermenter?
   Refer 14.5

2. Write a short note on GMO's in production of fermented foods.
   Refer 14.5

14.15 FURTHER READINGS

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MSc MICROBIOLOGY

MODEL QUESTION PAPER

MICROBIAL BIOTECHNOLOGY (36442)

Time: 3 Hours Maximum: 75 Marks

Part-A (10x2=20 marks)

Answer all questions

1. Fermentation
2. Metabolites
3. Extremophiles
4. Homokaryons
5. Pyrolysis
6. Transesterification
7. IPM
8. CPV
9. Virions
10. Protoplast

Part-B (5x5=25 marks)

Answer all questions choosing either (a) or (b)

11. (a) Mention some industrially important microorganisms with its applications (or)
    (b) Discuss on Somatic Hybridisation
12. (a) List out the biotechnological importance of algae (or)
    (b) Comment on Microprojectile Bombardment
13. (a) Explain about Electroporation (or)
    (b) Describe Nuclear Polyhedrosis Viruses
14. (a) What do you know about Siderphores?
    (b) Elucidate the structure of Baculovirus
15. (a) Discuss on the components of a biosensor (or)
    (b) Write about any two commercially important microbial polysaccharides with its importance

Part-C (3x10=30 marks)

Answer any 3 out of 5 questions

16. Write a brief note on scope of Microbial Biotechnology in food production
17. Mention the uses of SCP
18. Comment on biofuel production by algae
19. Elucidate *Bacillus thuringiensis* as Insecticide in detail
20. Comment on the impact of release of GMO into the environment.